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SINGLE CELL STUDIES OF NATURAL KILLER CELL IMMUNE SURVEILLANCE

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Single cell studies of natural killer cell immune surveillance
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To my family

ABSTRACT

Our immune system protects us from microbes such as viruses, bacteria and parasitic worms. The immune system also plays a key role in fighting cancer and in carrying out a function termed 'immune surveillance', natural killer (NK) cells patrol tissues and participate in the elimination of transformed cells and established tumors. By forming a tight contact to a abnormal cell such as a tumor cell, *i.e.* target cell, followed by secretion of toxic compounds the NK cell is able to kill the target cell. This process requires the formation of a lytic immune synapse which is a specialized interface formed between the NK cell and the target cell.

NK cell populations are heterogeneous as they are composed of individual cells that differ in their phenotype and functional responses. The work presented in this thesis aimed to study the functional heterogeneity within NK cell populations. In addition, we also set out to reveal what aspects of NK cell cytotoxicity that are influenced by the functional maturity, *i.e.* the level of education, of individual NK cells. Moreover, we were interested in identifying NK cells that are especially effective in killing tumor cells, so called 'serial killers'. The interest in serial killers is mainly due to their potential use in cancer therapies. For these purposes we have employed a microwell array system in combination with time-lapse imaging to perform long-term studies of the functional responses of individual NK cells. Furthermore, another aim was to determine the influence of the spatial distribution of ligands on NK cell responses. Here arrays of spatially separated ligands were patterned into artificial immune synapses (AIS) and NK cells interacting with AIS were followed using time-lapse imaging.

Results from the single cell analysis performed in the presented investigations has provided important insights to the dynamics of NK cell migration behavior and interactions with target cells. In **paper I** we characterized NK cells and found that these generally displayed a binary commitment as they were dedicated to a 'kill' or 'no kill' type of behavior. Furthermore, the results showed that NK cells can kill target cells via a fast or slow process which may have different effects on surrounding cells and tissues. In **paper II** we compared NK cells activated with the cytokine IL-2 to non-activated, resting NK cells in terms of their migration behavior, ability to form conjugates, and killing of tumor cells. The results showed that IL-2 activation of NK cells induces a migratory phenotype and enhances their ability to form conjugates and kill tumor cells. In **paper III** we compared the migration behavior, ability to form conjugates, and killing by NK cells at different levels of education. Here the results revealed heterogeneity in the migration behavior and cytotoxic response within the defined subsets of uneducated and educated NK cells. Still, the frequencies of NK cells that formed conjugates and killed target cells were significantly higher among educated NK cells compared to uneducated NK cells. In **paper IV** NK cells were imaged while interacting with ligands patterned into spatially separated AIS. We found that NK cells interacting with AIS composed of ligands for the activating receptors LFA-1 and CD16 displayed different morphologies and migration responses.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Immunsystemet eller immunförsvaret som det också kallas, skyddar oss mot de många olika typer av patogener som finns i vår omgivning såsom virus, bakterier och parasiter. De förödande konsekvenser som kommer av ett icke-fungerande immunsystem framgår tydligt av de återkommande infektioner som drabbar personer som lider av humant immunbrist virus (HIV).

Under pesten i Aten år 430 f.v.t. observerade filosofen och generalen Thucydides att personer som själva hade haft pesten kunde hjälpa till med att vårda de sjuka utan att återfå sjukdomen. Detta fenomen kan beskrivas med den latinska termen *immunis* vilket betyder 'fri' eller 'undantagen' som har gett upphov till det engelska ordet *immunity*.

Immunsystemet bekämpar dagligen infektioner med mycket smarta och komplicerade mekanismer och utrotar effektivt patogener, oftast utan att den drabbade individen ens märker av några symptom. Immunsystemet kan delas upp i det medfödda och adaptiva immunsystemet. Det medfödda immunsystemet förhindrar infektioner genom fysiska och kemiska barriärer samt immunceller. Exempel på barriärer som räknas till det medfödda immunsystemet är huden som bildar en fysisk barriär och den sura miljön i mage och tarmar som fungerar som en kemisk barriär.

När en infektion ändå får fäste tar det adaptiva immunsystemet över. Till det adaptiva immunsystemet tillhör så kallade T-celler och B-celler som kan vara specialiserade mot till exempel ett specifikt virus. Tillsammans kan populationer av T-celler och B-celler i en individ känna igen nästan vilken 'farlig' molekyl som helst.

Immunsystemet motarbetar också etablering av cancertumörer. En celltyp som bidrar stort i kampen mot cancer är specialiserade immunceller som kallas *natural killer* (NK) celler. NK-celler kan känna igen och döda sjuka celler, framförallt tumörceller men också virus-infekterade och stressade celler. NK-cellerna identifierar sjuka celler, som kallas *målceller*, via direkta cell-cell kontakter. Beslutet att döda eller inte döda en målcell baseras på vilka signaler NK-cellen får via sina receptorer som interagerar med molekyler på ytan av målcellen. Om signalerna är övergripande positiva så dödar NK-cellen målcellen genom att utsöndra giftiga ämnen. Under denna process skyddar molekyler på ytan av NK-cellen den själv mot effekten av dessa gifter.

Populationer av NK-celler består av olika celler vars funktionella styrka varierar. Alla NK-celler dödar inte tumörceller och NK-celler kan också uppvisa variationer i sitt beteende över tid. Många metoder som används för att mäta NK-cellers funktion tar dock inte hänsyn till denna variation, också kallad *heterogenitet*, inom populationer. Det behövs därför metoder som underlättar tidsmässigt långa studier av enskilda NK-celler. En sådan metod har utvecklats i vår forskningsgrupp där NK-celler och tumörceller hålls instängda i små brunnar (med en volym i storleksordning nanoliter) på ett mikrochip och som samtidigt filmas med ett mikroskop-system under ca 12 timmar. Brunnarnas mikroskopiska volym tillåter att små

populationer av celler kan studeras i varje brunn och på varje chip finns hundratals till tusentals brunnar. De filmer som skapas analyseras sedan i detalj för att ge svar på frågor som: Hur många tumörceller dödar varje enskild NK cell?, Hur snabbt migrerar varje NK cell?, Vilken typ av rörelsemönster uppvisar NK-cellen?, Vilken form och storlek har varje NK cell?

NK-celler som är extra effektiva när de dödar tumörceller, så kallade *serial killers* skulle kunna användas för olika typer behandlingar av cancer. Ett långsiktigt mål med studierna som presenteras här är därför att isolera och expandera populationer av NK celler som består endast av serial killers.

I den här avhandlingen presenteras fyra olika vetenskapliga artiklar. En första studie visade på att det finns en utspridd heterogenitet inom NK cells populationer, specifikt med avseende på deras individuella förmåga att forma kontakter med och döda tumörceller. Här påvisade resultaten att NK celler generellt kan delas upp i celler som dödar och celler som inte dödar tumörceller. Vad funktionen är hos de som inte dödar är oklart. Nästa studie gav insikter om vilka aspekter av NK cellers funktion som påverkas av aktivering med ämnen som kallas *cytokiner*. Det visade sig här att aktiverade NK celler rörde sig mycket mer än icke-aktiverade NK celler. Aktiverade celler var också bättre på att bilda kontakter och döda tumörceller.

I den tredje studien hittade vi betydelsefulla skillnader i beteendet och funktionen hos '*edukerade*' NK celler, vilket i princip betyder att de är funktionellt mogna, jämfört med de NK celler som inte är edukerade. Edukerade NK-celler verkar kunna växla mellan olika typer av rörelsemönster i större utsträckning än icke-edukerade NK celler, och är dessutom också bättre på att forma kontakter och döda tumörceller. Däremot visade resultaten ingen skillnad i sättet som edukerade och icke-edukerade NK celler dödade tumörceller.

Då NK celler formar kontakter med målceller bildas en *immunsynaps* mellan cellerna. I immunsynapsen arrangeras receptorer och molekyler i bestämda mönster. Syftet med den fjärde studien var att undersöka hur NK celler reagerar på att proteiner presenteras i olika sorters mönster som liknar de som uppstår i immunsynapsen. Mönster som bestod av aktiverande proteiner fördelade i rader av mikroskopiska prickar eller ringar trycktes på glasytor och vi filmade sedan NK celler som interagerade med mönstren. I dessa experiment observerade vi att två olika sorters proteiner, LFA-1 och CD16, hade motsatt effekt på NK cellernas migration. LFA-1 fick cellerna att migrera intensivt medan CD16 fick cellerna att stanna upp. Det visade sig också att NK cellerna formade mer långvariga och stabila kontakter på prickar jämfört med ringar.

Dessa resultat har bidragit till ökad förståelse för de olika beteenden som NK celler kan uppvisa i interaktioner med tumörceller. Vi har också kartlagt NK cellers olika rörelsemönster och hur dessa beror av cellernas aktiveringsgrad.

LIST OF SCIENTIFIC PAPERS

I. Bruno Vanherberghen, Per E. Olofsson, **Elin Forslund**, Michal Sternberg-Simon, Mohammad Ali Khorshidi, Simon Pacouret, Karolin Guldevall, Monika Enqvist, Karl-Johan Malmberg, Ramit Mehr, Björn Önfelt. Classification of human natural killer cells based on migration behavior and cytotoxic response. *Blood*. 2013 Feb 21; 121(8):1326-1334.

II. Per E. Olofsson, **Elin Forslund**, Bruno Vanherberghen, Ksenia Chechet, Oscar Mickelin, Alexander Rivera Ahlin, Tobias Everhorn, Björn Önfelt. Distinct Migration and Contact Dynamics of Resting and IL-2-Activated Human Natural Killer Cells. *Frontiers in Immunology*. 2014 Mar 7; 5:80.

III. **Elin Forslund**, Ebba Sohlberg, Monika Enqvist, Per E. Olofsson, Karl-Johan Malmberg, Björn Önfelt. Microchip-based single-cell imaging reveals that NK cell education via NKG2A regulates migration, target cell conjugation and probability of killing but not killing dynamics. *Manuscript, submitted.*

IV. **Elin Forslund**, Mattias Leino, Thomas Frisk, Per E. Olofsson, Björn Önfelt. Migration and morphology of human natural killer cells upon ligation of CD16 and LFA-1 on patterned immune synapses. *Manuscript.*

RELATED WORK NOT INCLUDED IN THE THESIS

I. **Elin Forslund**, Karolin Guldevall, Per E. Olofsson, Thomas Frisk, Athanasia E. Christakou, Martin Wiklund, Björn Önfelt. Novel microchip-based tools facilitating live cell imaging and assessment of functional heterogeneity within NK cell populations. *Frontiers in Immunology*. 2012 Oct 5; 3(300).

II. Monika Enqvist, Eivind Ask Häggernes, **Elin Forslund**, Mattias Carlsten, Greger Abrahamsson, Vivien Beziat, Sandra Andersson, Marie Schaffer, Anne Spurkland, Yenan Bryceson, Björn Önfelt, Karl-Johan Malmberg. Co-ordinated Expression of DNAM-1 and LFA-1 in Educated NK Cells. *Journal of Immunology.* Prepublished online 30 Mar 2015.

CONTENTS

LIST OF ABBREVIATIONS

AIS	Artificial immune synapses
CD	Cluster of differentiation
FACS	Fluorescence activated cell sorting
Fc	Fragment crystalline
FcR	Fc-receptor
IFN-γ	Interferon gamma
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
ILC	Innate lymphoid cell
ITAM	Immuno-receptor tyosine-based activation motifs
ITIM	Immuno-receptor tyosine-based inhibition motifs
IR	Inhibitory receptor
KIR	Killer-cell immunoglobulin-like receptors
LAMP-1	Lysosome-associated membrane protein-1
LFA-1	Lymphocyte function associated antigen-1
LIR	Leukocyte immunoglobulin-like receptors
LSCM	Laser scanning confocal microscopy
MACS	Magnetic-activated cell sorting
MSD	Mean-square displacement
μCP	Microcontact printing
MHC	Major histocompatibility complex
MTOC	Microtubule-organizing centre
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PDMS	Poly(dimethylsiloxane)
PMT	Photomultiplier tube
PVR	Poliovirus receptor
TCR	T cell receptor
TMAP	Transient migration arrest period

TNF-α	Tumor necrosis factor alpha
TLR	Toll-like receptor
SHP	(SH)2-containing protein tyrosine phosphatase

1 INTRODUCTION

This thesis presents investigations that employ novel techniques which facilitates studies of the dynamic functional properties of specific immune cells called natural killer (NK) cells. NK cells do not need any prior sensitization to perform their functions and are capable of clearing the body of abnormal cells such as tumor cells or virus-infected cells. By forming a tight contact to the abnormal cell, *i.e.* "target cell", and secreting toxic compounds the NK cell is able to kill the target cell.

Chapter 1 covers an introduction to some of the cells and mechanisms that make up the immune system and protects us from disease. Chapter 1 also includes a description of the function and biology of NK cells, this part focuses on human NK cells and their main effector functions as well as central concepts like NK cell education. Chapter 2 explains the techniques and specific microwell assays used in this thesis work. Chapter 3 presents the aims of the present investigations. Chapter 4 recaps the results from each paper and is followed by a discussion in chapter 5. Finally chapter 6 presents conclusions drawn and future perspectives.

1.1 AN INTRODUCTION TO THE IMMUNE SYSTEM

Our immune system protects us from microbes such as viruses, bacteria and parasitic worms. Microbes have throughout evolution caused human mortality and has consequently imposed a strong impact on natural selection (1). As a result, the wide diversity of microbes that surrounds us has facilitated the formation of our immune system into a remarkably complex and dynamic network of cells, tissues and processes. The immune system also plays a pivotal role in fighting cancer and in carrying out a function termed immune surveillance, the immune system eliminates transformed cells and kills established tumors (2). The immune system is absolutely essential for our survival, something that becomes evident by the increased susceptibility to infections and cancers of individuals infected with human immunodeficiency virus (3, 4).

The field of immunology disseminated out of the observation that individuals who had recovered from a certain infectious disease were afterwards able to nurse others back to health without again contracting the disease. This observation was first made by Thucydides during the plague of Athens in 430 BC and the Latin term "immunis" meaning free or exempt then gave rise to the English word immunity (5). The first recorded efforts attempting to induce immunity in order to protect individuals from infectious diseases occurred in the fifteenth century when crushed scabs of smallpox sores were inhaled or inserted into small cuts in the skin to protect from the disease (6). Later, in the eighteenth and nineteenth centuries, the work of Edward Jenner, Louis Pasteur and others laid the foundation for the development of modern vaccines that are today protecting us against many infectious diseases like diphtheria, measles, and polio (7). While the development of preventive vaccines is probably one of the greatest accomplishment of immunology and still an essential

field of research, the emergence of other novel immunotherapies, organ transplantations and preventive treatments require further understanding of the immune system. Basic research like the studies presented in this thesis contributes to the acquisition of new knowledge and a deeper understanding of the immune system which can be translated into applications, treatments, of novel immunotherapies.

In vertebrates the immune system can be divided into two parts: the innate and the adaptive immune system. During the course of evolution the innate immune system appeared before the adaptive immune system and some sort of innate immune system is likely present in all multicellular species (8). The actions of the adaptive immune system clears the body from microbes with great specificity and efficiency but acts more slowly, taking days to take effect, compared to the innate immune system which provides immediate protection against invading microbes.

1.1.1 Innate Immunity

The innate immune system defends the host against infection through anatomical barriers and cellular responses. Layers of epithelial cells make up the first line of defense against infection and are present in skin and line the mucosal and glandular tissues providing a physical barrier at the body's exposed surfaces. There are also chemical barriers that protects the body from microbes, including the acidic pH of the gut and specific enzymes and antimicrobial peptides found for example in secretions of saliva and tears (9). Another important component of the innate immune system is the complement system. The complement system is made up of a group of plasma proteins produced in the liver that contribute to eliminating microbes by causing lysis of microbes or infected cells, induce phagocytosis, and trigger inflammation.

Infections in epithelial layers and minor tissue damages such as a minor skin scrape may cause microbes or foreign substances to enter extracellular spaces and some microbes could also infect cells. Once infection has occurred, a cascade of inflammatory responses is triggered and the cellular effector mechanisms of the innate immune system are activated to clear the infection. Although conventionally said to act through non-specific recognition, more recent discoveries have shown that the innate immune system specifically detect and target most, if not all, microbes (10, 11). Cells of the innate immune system express pattern-recognition-receptors (PRRs) recognizing evolutionary conserved molecular structures called pathogen-associated molecular patterns (PAMPs) which are present on microbes but not on host cells (12). Toll-like receptors (TLRs) in vertebrates are a family of PRRs that have recently received much attention and in 2011 part of the Nobel Prize in physiology and medicine was awarded to Bruce Beutler and Jules Hoffmann for discovering the pivotal role of TLRs in activation of the immune response. Phagocytic cells like neutrophils and macrophages are efficient in clearing extracellular infections by ingesting and destroying microbes and cellular debris in a process called phagocytosis. Phagocytes can identify microbes through their PRRs as for example TLR4 that binds to lipopolysaccharide, a constituent of the cell wall of gram-negative bacteria and facilitates subsequent phagocytosis

of the bacteria (13). Phagocytes produce cytokines and chemokines that are small signaling molecules acting to recruit and activate more immune cells to help clear the infection.

During a viral infection proteins and nucleic acids from the virus can also be recognized by both extracellular and intracellular PRRs. NK cells have an important role in clearing viral infections as they are capable of identifying and killing virus-infected cells. Although innate mechanisms can efficiently eradicate microbes, many bacteria and viruses have developed strategies to subvert innate immunity by escaping recognition by PRRs or inhibiting innate signaling pathways and effector mechanisms (14).

In recent years, the group of innate lymphoid cells (ILCs) have been identified. This group of cells display functional heterogeneity and secrete many different cytokines that aid immune responses against virus, bacteria, and parasites (15). Although NK cells were discovered already in the 1970's, they are now considered to be cytotoxic ILCs (15, 16).

1.1.2 Adaptive Immunity

When the innate immune system is not able to alone clear the infection the adaptive immune system provides much needed mechanisms that specifically target the invading microbe. The main cells of the adaptive immune system are T cells and B cells which exert antigen-specific effector functions. T cells and B cells can be activated through interactions with dendritic cells which are antigen presenting cells belonging to the innate immune system. Dendritic cells thus function as a bridge between the innate and adaptive immune responses. While specializing in antigen presentation dendritic cells are also an early source of pro-inflammatory cytokines. Moreover, dendritic cells reside in peripheral tissues where they act as sentinels and upon activation they stimulate adaptive immune responses by presenting antigen to T cells and B cells (17). Receptors expressed by T cells and B cells are structurally similar and each clone (defined as the parent cell and its progeny cells) recognizes a specific antigen through a unique receptor. Recognition of that specific antigen initiates activation and proliferation where T cells and B cells produce numerous progeny cells that differentiate into effector and memory cell populations.

B cells carry membrane-bound antibodies and upon activation the B cell starts proliferating and the progeny cells differentiates into antibody-secreting plasma cells. Antibodies, also known as immunoglobulins (Ig), are proteins that specifically bind to an epitope on an antigen and enables clearance of that antigen. The total collection of antibody-specificities is extremely diverse and can target almost any kind of molecule including proteins, lipids, and polysaccharides. Antibody-mediated functions make up the humoral branch of adaptive immunity and include neutralization of microbes by for example binding and blocking molecules that microbes use to enter host cells. Neutralizing antibodies are very efficient in preventing the occurrence of infection and most preventive vaccines work by stimulating the production of neutralizing antibodies (18). Antibodies are composed of variable and constant structural domains where antigens bind to the variable regions which differ between clones

while the constant region is conserved between different clones and defines the antibody isotype or class. There are five different antibody isotypes: IgA, IgD, IgE, IgG, and IgM. The constant part of the antibody includes a fragment that crystallizes in solution and is therefore termed the fragment crystalline (Fc) region. Cell-mediated effector functions are facilitated by the interaction between the Fc-region and Fc-receptors (FcRs). Phagocytes can be activated through Fc-receptor interactions through opsonization where antibodies that have bound to a microbe stimulate phagocytosis of that microbe (Figure 1). Also NK cells express an Fc-receptor, FcγRIIIa (CD16), and are stimulated to kill antibody-coated cells and microbes in a process called antibody-dependent cell-mediated cytotoxicity (ADCC), as shown in figure 1. Furthermore, antibodies can activate the complement system. Initiation of the complement cascade may result in the formation of membrane attack complexes that punctures microbe membranes.

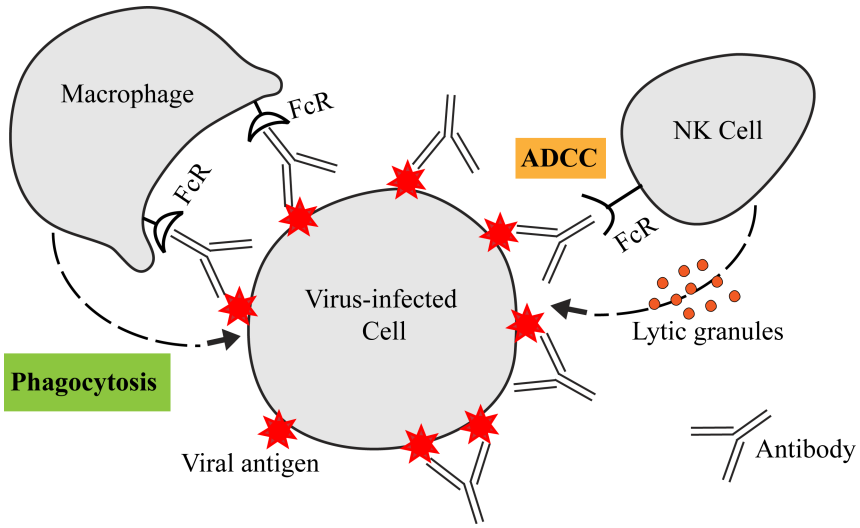

Figure 1. The Fc-regions of antibodies direct cell-mediated effector functions. Circulating antibodies bind to antigens on the infected cell and Fc-receptors expressed on the surface of the macrophage stimulates phagocytosis of the infected cell. Activating CD16 receptors expressed on the surface of the NK cell binds to Fc-regions on the antibodies and the interaction triggers NK cell cytotoxicity (ADCC) via release of cytolytic granule contents.

Over the course of infection B cells can go through a process called somatic hypermutation where the antigen-binding site of the antibody is modulated so that the affinity, that is the strength of the antigen-antibody bond, increases and supports a more effective humoral immune response. In addition, class-switching of antibodies changes the constant regions and can thus modulate and improve induced cell-mediated effector functions facilitating elimination of the particular invading microbe. Circulating memory B cells, that remain after the primary infection has been cleared, usually secrete somatically hypermutated and class-switched antibodies and are able to respond faster and stronger to a subsequent challenge with the same antigen. While B cells exert humoral immunity, the effector functions of T cells are cell-mediated and specialize in targeting infections caused by intracellular microbes. Each T cell expresses an antigen-specific receptor and when activated by antigen recognition and additional necessary stimuli it starts to proliferate. The progeny cells can be roughly divided into two types based on their chief function and expression of cell surface markers: 1)

$CD4^+$ helper T cells activate macrophages to destroy intracellular microbes and also secrete cytokines that regulate the activation and proliferation of other cell types including B cells, 2) $CD8^+$ cytotoxic T cells kill cells that contain microbes in their cytoplasm. While B cells can recognize many kinds of molecules, T cells mainly recognize peptides. Helper T cells bind peptides derived from extracellular bacteria that are presented in major histocompatibility complex (MHC) class II molecules on phagocytes. Cytotoxic T cells instead bind peptides derived from intracellular microbes that are presented in MHC class I molecules that are expressed by practically all cells in the body.

The effector functions carried out by T and B cells collaborate to eliminate microbes and a majority of the effector cells then die once the infection is cleared. However, some T cells and B cells persist, these are long-lived memory cells that will respond efficiently to a subsequent challenge with the same antigen.

1.2 FUNCTION AND BIOLOGY OF NATURAL KILLER CELLS

In the 1970's NK cells were first identified as immune cells capable of killing tumor cells in vitro without need for prior immunization. NK cells are widely distributed in tissues of mammals and can be found in the blood, spleen, liver, lung, bone marrow, lymph nodes, and uterus (19, 20). In human peripheral blood the fraction of lymphocytes made up of NK cells range from 2-18% (19). NK cells originate from CD34+ hematopoietic stem cells in the bone marrow and are thought to mature in secondary lymphoid tissues (21). In humans, NK cells are defined by their expression of the human neural-cell adhesion molecule CD56 and lack of expression of the T cell receptor (TCR) complex.

Unlike T cells and B cells that require antigen-specific recognition followed by a period of proliferation to mount an effective response, NK cells are ready to respond immediately upon activation and act as part of the first line of defense against pathogens. Functions of NK cells include direct cytotoxicity against tumor, virus-infected, and stressed cells (22, 23). The work presented in this thesis concentrates on their cytolytic function but NK cells also have important immunoregulatory functions. Recently much effort has been put into developing treatments of human malignancies in which the immune response is stimulated to eradicate tumor cells (24). Because of their naturally primed state in combination with their capability to kill tumor cells, NK cells are a suitable candidate for developing immunotherapy for cancer.

1.2.1 Cytokine production

In the early stages of viral infections, NK cells are the major source of interferon gamma (IFN-γ), a cytokine that stimulates macrophages to effectively destroy phagocytosed microbes. In return macrophages produce interleukin (IL)-12 that enhances NK cell cytotoxicity and induce further production of IFN-γ, thus NK cells and macrophages cooperate to clear intracellular infections. Moreover, IFN-γ is an important mediator in raising a potent immune response to viral infections and contributes to important processes

like activation of dendritic cells and priming of T cells. In addition, production of IFN-γ by uterine NK cells contributes to remodeling of decidual arteries during pregnancy (20, 25).
NK cells also produce other cytokines and chemokines that promotes inflammation and regulates recruitment and function of other hematopoietic other cells, as for example tumor necrosis factor alpha (TNF-α) that induces maturation of dendritic cells (26). Although typically considered to belong to the innate immune system, recent studies have shown that NK cells also have adaptive immune characteristics and can similar to T memory cells, respond rapidly upon secondary exposure to viral antigens (27). As mentioned previously, cytotoxic T cells can kill cells that have presented peptides from intracellular microbes in MHC class I molecules on their surfaces. Both virus-infected cells and tumor cells may however reduce their surface expression of MHC class I, thus allowing them to escape detection by cytotoxic T cells (28, 29). In such situations, NK cells offer a reserve recognition mechanism as NK cells instead of recognizing a specific peptide in MHC complexes, respond to the absence of a normal MHC class I expression, a feature called "missing self recognition" (30).

1.2.2 Cytotoxicity

NK cells circulate in blood and tissues and perform immune surveillance by scanning the surfaces of surrounding cells. The surface-bound receptors on the NK cell engage ligands on the target cell and the outcome of each cell-cell interaction is determined by a balance of activating and inhibitory signals received by the NK cells. This integration of activating and inhibitory input directs NK cell cytotoxicity against aberrant cells whereas tolerance for self is upheld as healthy cells are protected from NK cell attack (Figure 2).

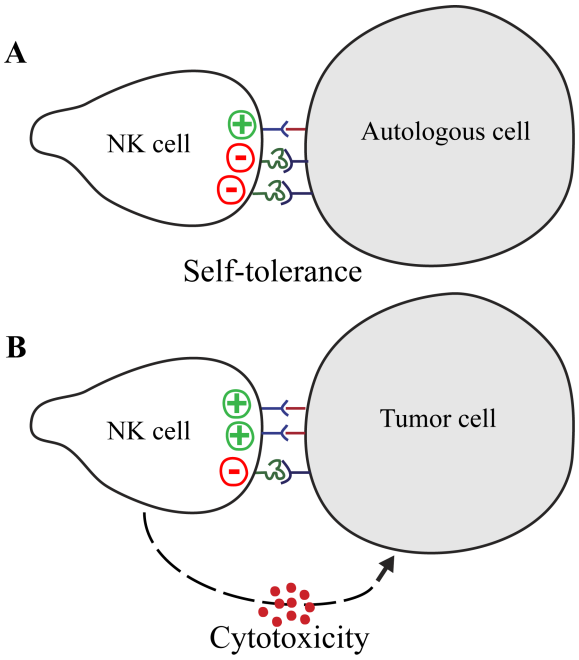

Figure 2. NK cells are regulated by a balance of inhibitory and activating signals (shown as minus and plus signs respectively). (A) Autologous cells that express normal levels of MHC class I molecules will be tolerated. (B) Tumor cells that display reduced expression of MHC class I molecules or express increased levels of activating ligands, will stimulate degranulation of cytotoxic proteins that induce target cell death.

1.2.2.1 Inhibitory and activating receptors and their ligands

NK cells can recognize a wide variety of ligands that are often polymorphic and many of the NK cell receptors are expressed in a stochastic manner, adding up to a diverse pool of potential receptor-ligand pairs with inhibitory or activating effects. Some of the inhibitory and activating receptor-ligand pairs discussed in this thesis are presented in table 1. The decision to kill a target cell will be taken when there is a lack of MHC class I molecules or an overexpression of activating ligands (31), as illustrated in figure 2. Whether a transplanted organ or tissue graft is accepted

or rejected by the immune system is mainly determined by the MHC, more specifically the MHC of the donor should match that of the recipient for the graft to be accepted. The genes that encode MHC molecules are present in various alleles and this polymorphism implies that any two people in an outbread population are unlikely to have identical sets of MHC genes. In humans, MHC class I molecules are named human leukocyte antigens (HLA), and are present on all healthy nucleated cells. There are six expressed HLA isotypes, of which HLA-A, -B, and -C are highly polymorphic whereas HLA-E, -G, and -F are conserved (32). The inhibitory receptor family of killer-cell immunoglobulin-like receptors (KIR), expressed on human NK cells, typically bind to the classical polymorphic MHC class I ligands, HLA-B and HLA-C, and the inhibitory CD94/NKG2A receptor binds to HLA-E, a non-classical MHC class I molecule. The level of HLA-E on the surface of a cell serves as an estimate of the overall expression of HLA molecules on that cell since its stability require binding of peptides derived form the leader sequence of other HLA molecules (33). The NKG2A receptor is also expressed in the mouse and the Ly49 family of inhibitory receptors in mouse functionally corresponds to KIR in humans. Although functionally equivalent, Ly49 and KIR receptors differ in their structure and have evolved independently (34). Different KIR specifically recognizes distinctive HLA alleles and the expression of KIR is variegated among NK cells present in one individual (35). The collected HLA-specificities of NK cells from one individual, mediated through KIR and NKG2A, is called repertoire.

Receptor name	**CD**	**Function**	**Ligand**
KIR	CD158	Most Inhibitory	HLA-B, HLA-C
NKG2A	CD94/CD159a	Inhibitory	HLA-E
LFA-1	CD11a/CD18	Adhesion, Activation	ICAM-1
FcγRIIIa	CD16	Activation	IgG
NKG2D	CD314	Activation	MICA, MICB, ULBP
2B4	CD244	Activation	CD48
DNAM-1	CD226	Adhesion, Activation	PVR, Nectin-2

Table 1. Human NK cell receptors.

Leukocyte immunoglobulin-like receptors (LIR) interact with a wide range of MHC class I molecules and LIR-1, also called Ig-like transcript 2, is expressed on subsets of NK cells (36). LIR-1/HLA interactions inhibit NK cell-mediated cytotoxicity but not to the same extent as KIR or NKG2A engagement (37). NK cells also express other inhibitory receptors that bind to non-MHC class I ligands although the importance of these as well as LIR-1 in regulation of NK cell function remains to be resolved (38).

The known receptors that inhibit human and mouse NK cells generate inhibitory signals via tyrosine-based inhibition motifs (ITIM). Receptor-ligand interactions leads to tyrosine-phosphorylation of ITIM and recruitment of SHP-1 or SHP-2 phosphatases (35). Engagement of inhibitory receptor generates signals that interfere with activating signals and consequently

inhibit NK cell cytotoxicity and cytokine production. Activating NK cell receptors, including activating KIR, CD94/NKG2C, CD16, NKp30 (CD337), and NKp46 (CD335), associate with adaptor proteins that contain immuno-receptor tyrosine-based activation motifs (ITAM) and the receptor-ligand interactions results in activating signaling through recruitment of tyrosine kinases Syk and Zap-70 (31, 38). The low-affinity activating Fc-receptor CD16 binds to the Fc-portion of IgG enabling NK cells to kill antibody-coated cells and microbes through ADCC. Activating receptor signaling in generally results in calcium flux which is due to a rapid release of Ca^{2+} from the endoplasmic reticulum and entry of Ca^{2+} through ion channels in the plasma membrane (39).

Activating KIR and NKG2C are closely related to inhibitory receptors. The structure of the extracellular domains of activating KIR are very similar to that of their corresponding inhibitory receptors and although it has been shown that some activating KIR recognize HLA molecules, the ligand specificity of many activating KIR remains to be determined (40). The functional implications of activating KIR and whether these receptors actually have HLA molecules as their primary ligands, are intriguing questions yet to be answered. CD94 can form a heterodimer with either NKG2A or NKG2C and both isoforms bind to HLA-E (33). While the cytoplasmic domain of inhibitory receptors contains ITIM, activating KIR and NKG2C instead signal via ITAM-containing adaptor protein DAP12 (41, 42). Like activating and inhibitory KIR, also NKG2A and NKG2C also share the same ligand although inducing opposing inhibitory and activating signals. Recognition of HLA-E by both NKG2A and NKG2C has been indicated to be peptide-dependent (43), and this was demonstrated in a study showing that the heatshock protein hsp60, which is induced by cellular stress, forms a complex with HLA-E which is not recognized by NKG2A or NKG2C (44).

1.2.2.2 The lytic immune synapse

Cytotoxic T cells and NK cells share several functional and phenotypic features and both cytotoxic T cells and NK cells can kill target cells through directed exocytosis of cytolytic granules. Although NK cell can also exert cytotoxicity via death receptors and ligands (45), NK cell-mediated lysis of tumor and virus-infected cells is mainly mediated via lytic granule degranulation (46). This process requires the formation of a lytic immune synapse, a specialized interface formed between two cells where at least one of them is an immune cell (47). The lytic immune synapse is formed in distinct stages initiated by contact and adhesion to the target cell, followed by reorganization of the cytoskeleton, polarization and degranulation of lytic granules into the synaptic cleft, and finally termination (48, 49). It was first described for cytotoxic T cells and later for NK cells and other immune cells. In immune synapses formed by T cells, proteins have been shown to segregate into two distinct regions. The TCR accumulate at the center of the synapse in a cluster called the central supramolecular activation cluster (cSMAC) surrounded by a second region, the peripheral supramolecular activation cluster (pSMAC) that includes LFA-1 (50).

Although both cytotoxic T cell and NK cell are able to kill target cells through degranulation, these different cell types have important features that distinguish them from one another and

accordingly the process of immune synapse formation should not be assumed to be identical in T cells and NK cells. Unlike naïve T cells, NK cells carry preformed lytic granules setting them in an "armed-and-ready" state that requires more tight control of the process of lytic granule degranulation that leads to target cell killing (48, 49, 51).

The formation of an immune synapse requires adhesion facilitated through integrins, in particular LFA-1, which is expressed by all leukocytes. LFA-1 can be presented in an inactive or active conformation on the cell surface and active LFA-1 binds to intercellular adhesion molecule-1 (ICAM-1) on the target cell, an interaction that facilitates strong adhesion and induces clustering of LFA-1 and polarization of lytic granules towards the immune synapse (52, 53). About 20% of NK cells in a resting state and 60-80% of IL-2 activated NK cells bind to plate-bound ICAM-1 and to insect cells expressing ICAM-1 in the absence of any other activating ligands (54). Thus, LFA-1-mediated adhesion of NK cells to target cells can occur prior to engagement of activating receptors and ~10% of $CD56^{dim}$ NK cells display the active form of LFA-1 on their surface in a resting state (55). Still, engagement of activating receptors such as CD16, NKG2D (CD314), 2B4 (CD244), DNAM-1 (CD226), and LFA-1 itself, leads to inside-out signaling resulting in a conformational change of LFA-1 from an inactive to an active form, and thus supports firm adhesion (55). In contrast, inhibitory signals form KIR and NKG2A can hamper inside-out signaling and thus obstruct LFA-1-mediated adhesion to target cells (53, 55, 56).

The binding of LFA-1 initiates immune synapse formation and induces reorganization of the actin cytoskeleton (49, 53, 57). Talin, a cytoskeletal adaptor protein, is necessary for both inside-out activation of LFA-1 and outside-in signaling resulting from LFA-1 ligation (57). Subsequent of LFA-1 ligation and initiation of synapse formation, LFA-1, actin and talin cluster and form a ring in the pSMAC at the periphery of the synapse, and a complex of

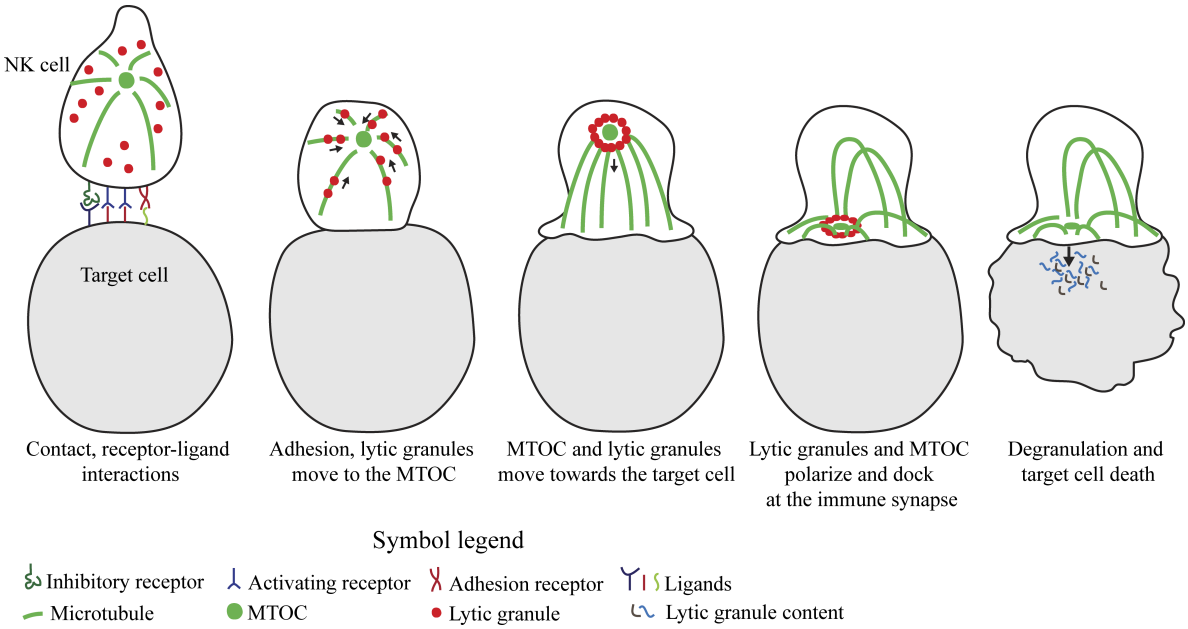

Figure 3. Polarization and degranulation of lytic granules at the immune synapse. Ligation of adhesion and activating receptors initiate formation of a lytic immune synapse. Lytic granules move along microtubules and converge at the MTOC. Next, the MTOC and lytic granules polarizes to the immune synapse where the content of the lytic granules are released and cause target cell death.

multiple signaling molecules including (SH)2-containing protein tyrosine phosphatase-1 (SHP-1) assembles in the cSMAC in the center of the synapse (58). It was previously believed that actin was completely cleared from the center of the synapse where the lytic granules are secreted, but recent use of super-resolution microscopy has revealed that lytic granules are secreted through an actin-mesh (59, 60).

Following reorganization of actin filaments, the microtubule-organizing center (MTOC) and lytic granules moves towards the synapse (Figure 3). Before this polarization occurs, the lytic granules move rapidly along microtubules via dynein/dynactin motor proteins and converge at the MTOC (61). What exact molecules and mechanisms that causes the MTOC and lytic granules to polarize towards the synapse is not known. However, it has been shown that in NK cells the minus-ended directed motor protein kinesin-1 contributes to this process (62). Polarization of the MTOC and accompanying lytic granules does not only occur when the NK cell is committed to killing the target cell, but can also occur in non-cytolytic conjugates (61). Outside-in signaling through LFA-1 is alone sufficient for actin polarization and lytic granule polarization although polarization of lytic granules can also be induced in the absence of LFA-1, through concurrent engagement of the activating receptors CD16 and 2B4 (38, 53).

Moreover, engagement and clustering of LFA-1 and polymerization of actin at the synapse acts in synergy to sustain firm adhesion to the target cell leading to NK cell flattening and spreading, resulting in an increased synapse diameter (48, 51). Here tethering of the ligand is important as ICAM-1 needs to be immobilized in the target cell membrane to interact properly with LFA-1 (63). Activating receptor-ligand interactions, specifically the 2B4 receptor interacting with CD48 on the target cell, may further strengthen adhesion to the target cell (54).

The process of degranulation involves docking of lytic granules at the plasma membrane, which is dependent on Rab27a and MUNC13-4 recruitment (64). Next, soluble nsf attachment protein receptor (SNARE) proteins enable fusion of lytic granules with the plasma membrane resulting in release of perforin and granzymes into the synaptic cleft (48). Perforin forms transmembrane channels in the target cell membrane that facilitates cell lysis and delivery of granzymes into the cytoplasm of the target cell where they induce a process of programmed cell death, apoptosis (65, 66). The NK cell is itself protected from the degranulated cytotoxic proteins, a mechanism that seems to be at least partially dependent on the exposure of lysosome associated membrane protein-1 (LAMP-1, CD107a) on the surface of the NK cell (67).

1.2.3 Phenotypic and functional heterogeneity in NK cell populations

Because the function of NK cells is controlled by inhibitory and activating signals, the phenotypic variation in surface expression of inhibitory and activating receptors is closely linked to the functional heterogeneity. Two main subsets of human NK cells have been identified that differ in their phenotype, tissue localization, maturity, and functionality: 1) $CD56^{bright}CD16^{dim/neg}$ ($CD56^{bright}$) NK cells and 2) $CD56^{dim}CD16^{bright}$ ($CD56^{dim}$) NK cells.

The majority of NK cells found in peripheral blood (>95%) belong to the cytotoxic $CD56^{dim}$ subset while $CD56^{bright}$ NK cells are generally not cytotoxic and mostly reside in secondary lymphoid organs where they produce cytokines (68). In a resting state, $CD56^{dim}$ but not $CD56^{bright}$ NK cells, express KIR and carry perforin-containing lytic granules (69). However, an excessive number of other subsets of human NK cells can be defined based on their surface expression. Remarkably, the use of mass cytometry has revealed that there are tens of thousands phenotypically distinct NK cell subsets present in human peripheral blood, of which the functional diversity is poorly understood (100).

1.2.4 Influence of cytokines on effector functions

In order to produce an environment *in vitro* that mimics an inflammatory setting *in vivo*, IL-2 and other cytokines can be used to stimulate NK cell cytotoxicity, cytokine secretion, and proliferation. During an infection, the activity of NK cells is heightened by cytokines like IL-15, IL-12, and type I interferons. These soluble factors augment the cytolytic function of NK cells and are secreted by phagocytes that have encountered microbes. T cells produce IL-2 and T cell-derived IL-2 can together with IL-12 costimulate IFN-γ secretion by $CD56^{bright}$ NK cells residing in lymph nodes (70). Moreover, it has been shown that the availability of IL-2 regulates NK cell cytotoxicity and that IL-2 stimulation promotes target cell conjugation (71). This improved ability to form conjugates is at least partially due to cytokine induced upregulation of adhesion molecules. Treatment with either IL-2 or IL-15 upregulates the surface level of LFA-1 on NK cells, which strongly augments LFA-1-mediated adhesion to target cells (54). Furthermore, IL-2 activated NK cells can be stimulated to degranulate and kill target cells in response to stimulation of any of the following activating receptors alone, CD16, Nkp46, NKG2D, 2B4 and DNAM-1 while resting NK cells, with the exception of activation through CD16, seem to require concurrent stimulation of several activating receptors giving an synergistic effect that triggers cytotoxicity (72). Therefore IL-2 activated NK cells appear to be primed to form conjugates and exert cytotoxicity in a less controlled process than resting NK cells.

1.2.5 Development, education and self-tolerance

Although it is known that NK cells derive from bone marrow hematopoietic $CD34^{+}$ stem cells, details on the developmental path of NK cells is still unclear (21). NK progenitor cells can be found in extramedullary tissues and the maturation of progenitor cells into mature NK cells probably occur in secondary lymphoid organs (21). Bone marrow originating stem cells differentiate into $CD56^{bright}$ NK cells which are precursors of $CD56^{dim}$ NK cells. This developmental path is supported by the observation that $CD56^{bright}$ NK cells have longer telomeres than $CD56^{dim}$ NK cells and also by the sequential appearance of $CD56^{bright}$ and $CD56^{dim}$ NK cells in peripheral blood subsequent of bone marrow or stem cell transplantations (21, 68, 73). $CD56^{dim}$ NK cells that initially express NKG2A seem to further differentiate and lose their expression of NKG2A and successively acquire expression of KIR. Acquisition of NKG2A and KIR expression is associated with a reduced proliferation capacity (74, 75). The CD57 antigen is a terminally sulfated glycan carbohydrate epitope

(76). Expression of CD57 is considered a T-cell marker indicating replicative senescence, *i.e.* inability to undergo new cell-division cycles (76, 77). Furthermore, expression of CD57 on $CD56^{dim}$ NK cells is associated with a reduced proliferation capacity suggesting that CD57 expression is a marker of late stage differentiation in NK cells (74).

Apart from the process that leads to developmental maturity, NK cells go through another process to reach a state of functional maturity, this process is commonly referred to as "education" or "licensing" (78-80). Functional maturity is largely measured by the level of cytotoxicity against susceptible target cells or cytokine production. Responses of educated NK cells are generally stronger than that of uneducated, hyporesponsive NK cells. A recent study showed that human KIR^- NK cells and murine NK cells from MHC class I deficient mice formed less stable conjugates with target cells, as compared to whole NK cell populations from humans and wild-type mice (81). The reduced ability of unlicensed NK cells to form stable conjugates was attributed to impaired inside-out signaling to LFA-1 by activating receptors (81). Moreover, it was recently found that educated NK cells have better survival as expression of NKG2A or self-KIR correlates with a reduced incidence of apoptosis during stress (75). As discussed previously, inhibitory receptors that bind to self-MHC molecules are responsible for protecting healthy autologous cells against damaging effects of NK cell activity. Although the mechanism of education has not yet been fully elucidated, there is a general agreement that interactions of inhibitory receptors with their cognate MHC class I ligands are required for NK cells to become educated. This conclusion stems from the reduced responsiveness displayed by human and mouse NK cells that have developed in a MHC-deficient host, or by NK cells that lack expression of inhibitory receptors binding to MHC molecules expressed by the host (78, 80, 82-86). The education process tunes the responsiveness of NK cells and the functional potency of individual NK cells depend on the quantity and quality of signaling received through inhibitory receptors (79, 87). Furthermore, the variegated expression of inhibitory receptors introduces a functional heterogeneity within NK cell populations. In human peripheral blood there are hyporesponsive, uneducated $NKG2A^-KIR^-$ NK cells that lack expression of educating inhibitory receptors, as well as NK cells that lack expression of cognate KIR (78, 88, 89).

1.2.5.1 Role of cytokines in development and education

Cytokines influence the function, development and phenotype of NK cells. Most NK cells express a heterodimeric IL-2 receptor, IL2Rβγ, which has intermediate affinity for IL-2 (68). Stimulation with IL-2 or IL-15, both signaling through the IL-2 receptor, enhances the cytolytic activity of $CD56^{dim}$ NK cells but has a lesser effect on the proliferation of these cells (73, 90, 91). On the other hand $CD56^{bright}$ NK cells express the high-affinity IL2Rαβγ receptor and can in response to IL-2 be prompted to proliferate robustly, acquire expression of KIR and perforin, and mature into cytotoxic $CD56^{dim}$ NK cells (69, 73, 90, 92). Furthermore, $CD34^+$ hematopoietic progenitor cells expressing the high affinity IL-2 receptor have been shown to be able to differentiate into $CD56^{bright}$ NK cells in response to both IL-2 and IL-15 stimulation (93). Still, the IL-2 gene is not expressed by bone marrow stromal cells

and NK cells are able to develop in IL-2 deficient mice, thus IL-2 probably does not have any profound role in NK cell development in vivo (94, 95). In contrast, IL-15 appears to be important for NK cell development (94).

The difference in functional potency observed between educated and uneducated NK cells in a resting state can be abolished through cytokine stimulation. Culture of uneducated human NK cells in the presence of cytokines reverses the hyporesponsive state and induces acquisition of both effector functions and expression of educating inhibitory receptors (88, 96). Furthermore, NK cells that have developed in a human or mouse that have a significantly reduced level of MHC class I expression, are hyporesponsive in a resting state but can become responsive after IL-2 culture (80, 84, 86, 97, 98). As the effector functions of NK cells are potentially self-destructive, it is crucial for the host that NK cells are self-tolerant. Therefore the anergic or hyporesponsive state of NK cells that lack receptors for cognate MHC class I molecules is protecting autologous cells from NK attack. Yet the hyporesponsive state can be reversed by cytokine activation, indicating a role for these NK cells during inflammatory conditions and indeed recent studies have provided evidence that hyporesponsive, uneducated NK cells have a significant role in clearing bacterial and viral infections (83, 99). During such conditions, uneducated NK cells could be helpful in clearing infections as they are not inhibited by expressed MHC class I and thus may kill infected cells efficiently.

2 METHODOLOGICAL CONSIDERATIONS

The work presented in this thesis has involved the use of conventional techniques like flow cytometry and also more novel approaches to single cell analysis employing the microwell-chip platform and related analytical tools developed in our research group. All the presented examinations in papers I through IV deal with studies of human NK cells isolated from peripheral blood of healthy donors.

2.1 FLUORESCENT LABELING

Cell-surface molecules as well as structures contained within cells *e.g.* the cytoskeleton can be detected by labeling with antibodies that have been conjugated to fluorophores. These antibodies bind specifically to epitopes on intracellular or surface-expressed molecules and the linked fluorophores absorb light of a specific wavelength range, and in turn emit light of a longer wavelength (*i.e.* of a different color) as illustrated in figure 4.

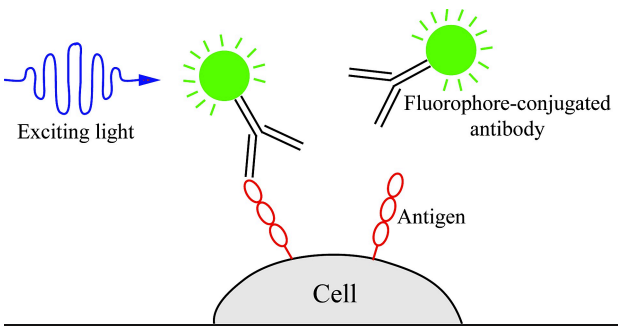

Figure 4. Illustration of a fluorophore-conjugated antibody bound to an antigen expressed on a cell. The fluorophore absorbs light (blue) and emits fluorescence (green) facilitating visualization of the labeled protein.

Apart from fluorophore-conjugated antibodies, there are many other kinds of fluorescent markers that are capable of

recognizing specific molecules or cellular structures. Furthermore, fluorescent proteins like green fluorescent protein (GFP) can be incorporated into the genome in such a way that GFP is expressed together with the protein of interest, thus functioning as a reporter of expression.

Different protocols for labeling cells with fluorescent markers can be used depending on whether the cells are alive or fixed (dead) at the time of staining. If the purpose of the experiment is to visualize intracellular structures at a single time point, then the cells can be fixed and permeabilized prior to staining. Fluorescent labeling of the cytoskeleton in NK cells, using antibodies against tubulin and actin, was performed this way in paper IV. In the microwell-based studies presented in this thesis (papers I-III), living cells were labeled with acetoxymethyl (AM) ester derivatives of fluorescent indicators called calcein AM (Life Technologies). These are cell-permeant dyes that function as fluorescent cell tracers and indicators of viability. Calcein AM dyes freely diffuse into living cells and once inside the cell it is cleaved by esterases resulting in a charged form of the molecule which leaks out of the cell considerably more slowly than the rate by which it entered the cell. If the plasma membrane is damaged or ruptured, as it may be when the cell dies, the calcein dye rapidly leaks out of the cell. As a complement to calcein labeling, target cells were also labeled with the fluorescent cell tracer dye DDAO which contains succinimidyl ester reactive groups that bind to amines present on intracellular proteins and other biomolecules. Thus upon target cell death, the intensity of the calcein staining decreases while the DDAO staining remains.

Fluorescent markers may also change their fluorescent properties depending on the milieu, as for example the calcium-indicator Fluo-4 from which the emitted fluorescence increases with rising levels of Ca^{2+}. Hence, the fluorescence intensity emitted from cells loaded with Fluo-4 is proportional to the intracellular concentration of Ca^{2+}. In paper IV, NK cells were stained with Fluo-4 in order to evaluate the level of intracellular Ca^{2+} which rapidly increases upon ligation of activating receptors like CD16.

2.2 IMAGING TECHNIQUES

Simple optical microscopes magnifies images of small specimens and are used as an every-day tool in laboratories to for example check the status of cells growing in cultures. Complicated experiments may however require more advanced optical microscopes, so called imaging systems, that allow for acquisition of high-resolution images with confocal microscopy, or time-lapse imaging of living cells and tissues. Both types of imaging techniques have been employed in projects presented in this thesis.

2.2.1 Fluorescence microscopy

Fluorescent labeling followed by analysis using a fluorescence microscope enables detection and analysis of spatially separated intracellular compartments and molecules. In fluorescence microscopy, the specimen is illuminated by a light-source of a wavelength that is absorbed by fluorophores in the specimen. Filtering of both the exciting light and the emitted light facilitates detection of several distinct fluorophores in separate channels. The emitted light can be detected by sensitive area detectors, such as in video-cameras, that form an image

directly. By overlaying images from several channels, each displaying the emitted fluorescence of a specific fluorophore, the resulting multi-colored image shows the relative spatial localization of for example cells or proteins labeled with fluorescent probes, as shown in figure 5.

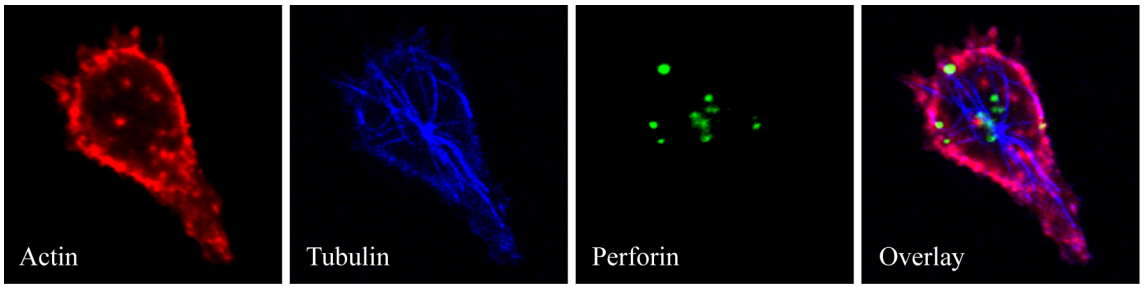

Figure 5. Confocal images of a NK cell stained with fluorescent phalloidin that binds to actin filaments (red), and fluorophore-conjugated antibodies for tubulin (blue), and perforin which is contained inside lytic granules (green).

2.2.2 Laser scanning confocal microscopy

Images that were acquired with fluorescence microscopy can be unclear due to the contribution of fluorescent molecules that are located above and below the focal plane. This problem has been solved in laser scanning confocal microscopy (LSCM) through the use of an adjustable pinhole that prevents detection of any light that does not come from the imaged focal plane. In LSCM, the specimen is scanned point-by-point with a laser beam and the emitted fluorescence is detected and converted into an image through point detectors as for example photomultiplier tubes (PMTs) and photodiodes. Through sequential imaging of focal planes in the specimen, so called optical sectioning, and subsequent combination of the images from each focal plane it is possible to create a projection of the specimen in three dimensions. By using LSCM instead of conventional fluorescence microscopy, the resolution increases both the lateral x- and y-directions, as well as axially in the z-direction. Because of the improved resolution in the lateral plane, LSCM is suitable to use not only to obtain three-dimensional images but also for acquiring images in a single focal plane.

2.2.3 Time-lapse imaging

Time-lapse imaging is suitable for studies of dynamic cellular events, and in terms of the work presented in this thesis, it was used to record the functional response and behavior of NK cells. Images are acquired at predetermined intervals and when played, a video is produced. Live cell imaging over extended periods of time requires special precautions - the imaging setup must consider that overexposure of light is harmful to living cells, especially in the presence of (101). Therefore, the light-exposure should be minimized and images should thus be acquired with fast scanning speed using a laser set at low power. Apart from light-exposure, other parameters like the temperature, humidity, and pH also influences the functional state of imaged cells, and must thus be kept at physiological levels to sustain cellular viability and function.

2.3 TARGET CELL LINES

Target cell lines used in this work included the human leukemia K562 cell line and the adherent human embryonic kidney HEK293T cell line. The K562 cell line was derived from cells from a patient suffering from chronic myelogenous leukemia in 1975, and the K562 cell line was later found to be of erythroid origin (102, 103). The HEK293 cell line was derived from human embryonic kidney cells through transformation with fragments of an adenovirus in the 1970's, although HEK cell lines have later been shown to share characteristics with neuronal cells (104, 105). The expression levels of MHC class I is reduced on HEK293T cells and K562 cells lack expression of MHC class I. Thus, both these cell lines are susceptible to NK cell cytotoxicity. The K562 cell line is particularily sensitive to NK cell-mediated lysis and has been widely used as a target cell line to study functional responses of NK cells.

2.4 SINGLE CELL TECHNOLOGIES

Immune cell populations are heterogeneous meaning that the individual cells that make up a population of cells may differ in their phenotype and functional responses. Measurements at the population-level offers an assessment of the average response, neglecting that two cells from the same population may exert different characteristics. Thus, when studying immune cells the employed analytical tools should in addition to evaluating the average response of the population, preferably also allow for assessment of individual cells.

2.4.1 Flow cytometry and related techniques

Flow cytometry is one of the most frequently used analytical techniques within the field of immunology and enables single cell analysis. Cells in suspension are introduced inside a flowing column of sheath fluid facilitating hydrodynamic focusing of single cells into a narrow stream (Figure 6). The flowing cells then pass through a laser beam and the scattered light is measured in both in the direction of the laser beam (forward scatter, FSC) and at a 90° angle (side scatter, SSC). FSC implies the size of individual cells while SSC indicates the granularity which increases with the amount of membrane-enclosed structures contained within the cell.

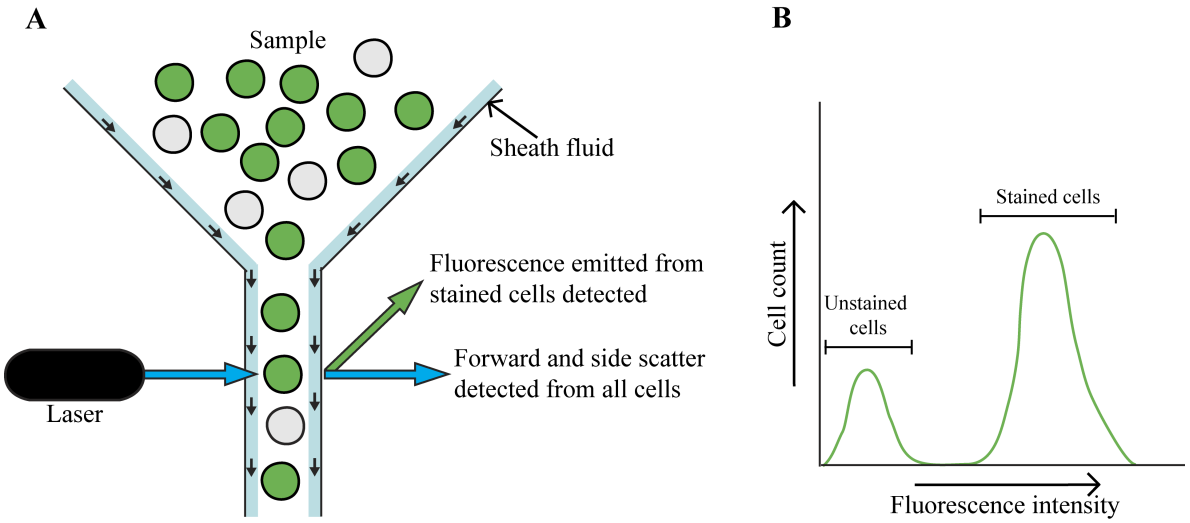

Figure 6. Basic principle of flow cytometry. (**A**) A mixture of cells pass the laser beam causing scattering of light and emission of fluorescence. Scattered light from all cells as well as the color and intensity of fluorescence emitted from labeled cells is detected. (**B**) The fluorescence intensity visualized as a histogram where unstained and stained populations of cells can be identified.

Flow cytometry is used to assess the expression of molecules that have been labeled with fluorophore-conjugated antibodies or other fluorescent probes. A flow cytometer can be equipped with several lasers of different wavelengths facilitating detection of various fluorophores. As the cells pass through the laser beam, the light emitted from each cell is filtered and recorded by a series of PMTs. Filtering of the emitted light before it reaches the PMTs enables signals from different fluorophores to be detected in different channels. Each cell that pass the laser is counted by the flow cytometer and the wavelength and intensity of its emitted fluorescence is recorded. The foremost advantage of flow cytometry is the efficiency by which large quantity of data can be acquired as thousands of individual cells can be measured within just seconds. Flow cytometry is generally not appropriate for studies of dynamic events, as for example cell migration. Furthermore, it is not possible to assess the spatial localization of proteins and structures on or within single cells using conventional flow cytometry. However, a novel analytical flow-based technique called imaging cytometry integrates fluorescence microscopy with flow cytometry thus overcoming this limitation as it produces images of single cells in a high-throughput manner. Still, once the cells have passed through the laser they are discarded, thus presenting a major disadvantage as it is not possible to follow properties and behavior of single cells over time.

The flow cytometer can however be modified to allow for fluorescence activated cell sorting (FACS), where one or several populations of cells of specific phenotypes are collected to allow for further studies. Here, fluorescence-labeled cells pass through a laser beam and subpopulations are sorted based on the characteristics of the scattered light and the emitted fluorescence. Accordingly, FACS facilitates sorting of living cell populations of interest based on for example their expression levels of surface-bound molecules. In paper III, FACS was used to sort specific subsets of NK cells for subsequent studies of their functional responses using both imaging techniques and flow cytometry.

2.4.1.1 CD107a degranulation assay

Expression of CD107a is upregulated on the surface of NK cells that have been stimulated with MHC-deficient target cells and CD107a expression correlates with both NK cell-mediated lysis and cytokine secretion (106). Moreover, upon polarization of lytic granules CD107a colocalizes with perforin and the cell-surface expression of CD107a on NK cells in conjugates correlates with target cell death (52). In paper I and III, the expression of CD107a was measured in order to evaluate the fraction of cytotoxic NK cells within a population. In these experiments, NK cells and target cells were incubated together for 4 hours in the presence of fluorophore-conjugated antibodies for CD107a, the relative expression of CD107a was subsequently measured by flow cytometry.

2.4.2 Microwell chip assays

Integration of bioanalytical methods with microfabrication techniques has lead to the development and use of miniaturized assays and microfluidics in biological research. Miniaturized assays such as the microwell assays used in this thesis work are favorable in studies where only small samples can be obtained. Also the required volumes of expensive reagents and associated costs are scaled down compared to conventional assays. Moreover, the use of small volumes (<µl scale) makes microfluidic systems appropriate for single cell analysis and studies of cell-cell interactions. Also the distances between cells in a microfluidic system are short, thus time-to-interaction resembles physiological settings.

Much of the work presented in this thesis has involved time-lapse imaging of NK cells and tumor target cells co-incubated in microwells. Time-lapse imaging facilitates studies of dynamic events like NK-target cell interactions and migration. Conventional approaches aiming to perform time-lapse imaging of single cells are often faced with difficulties as cells easily move out of the field of view. Our research group and collaborators have developed miniaturized assays where the studied cells are confined in microwells, enabling us to follow them over extended time periods (107-109).

Other groups have also developed comparable microwell array systems for imaging-based single cell studies. Microwell-chips composed of poly(dimethylsiloxane) (PDMS) have been used to study functional responses of single NK cells and cytotoxic T cells (110, 111). Similar PDMS microwell array systems have also been evaluated in our research group but compared to the currently used silicon microwells, it was found to be less suitable for use in longer experiments (>4 hours) (107).

In the present investigations, silicon microwell-chips containing arrays of wells of side dimensions 650×650, 450×450 or 50×50 μm^2 were used. The larger microwells were used for studies of migration and killing while the smaller microwells were used only to study killing. Because they are adherent and stationary, the HEK293T cells were used as target cells when migration was studied along with killing while K562 target cells were used in smaller microwells when only killing was examined.

2.4.2.1 Microwell-chip fabrication

The microwells are contained in arrays on silicon chips bonded to a glass bottom to facilitate imaging with an inverted microscope. Fabrication of the microwells was achieved through standard microfabrication techniques where a silicon wafer was exposed to alternating periods of etching and side-wall passivation. Vertical deep reactive ion etching with with SF_6 'eats' through the silicon wafer while passivation involves deposition of C_4F_8, a chemically inert fluorocarbon layer that protects the wafer from etching in the horizontal direction. Next, the structured silicon wafer was permanently bonded to a glass through the process of anodic bonding. Here, chemical bonding of the silicon wafer to the glass was accomplished by clamping the two substrates together and applying a strong electrical field at around 400°C.

2.4.2.2 Priming the microwells for experiments

In preparation for experiments, the chip was fixed into a holder and a gasket and a lid was placed on top. The gasket was placed between the chip and the lid to prevent fluid leakage, magnets mounted into the lid fixed it to the holder. Because air is trapped in the microwells when fluid is added to the dry microwell-chip, the microwells were initially filled with buffer through degassing. In preparation for migration studies, the microwells were coated with fibronectin to provide a substrate that supports migration of NK cells (112). The microwells were then filled with cell culture medium, with a reservoir of medium above the wells.

2.4.3 Patterning of artificial immune synapses

The formation of an immune synapse involves segregation of receptor-ligand pairs into micro-sized clusters (described in section 1.2.2.2). Protein patterning techniques facilitate spatial segregation of ligands into discrete areas on a substrate. This way, receptors on immune cells can be directed into predetermined patterns and resulting cellular responses may be recorded. Some previous studies have employed ligand-patterning techniques in order to create synthetic immune synapses and study functional responses of T cells and NK cells (113-116).

In paper IV, we have patterned antibodies for the activating NK cell receptors, CD16 and LFA-1, and recorded the responses of individual NK cells interacting with these discrete areas of stimuli called artificial immune synapses (AIS). In a couple of experiments, ICAM-1 was patterned instead of anti-LFA-1. Furthermore, by creating surfaces with alternating areas of activating ligands and areas empty of stimuli we could study the effect of CD16 and LFA-1 receptor ligation on the motility and morphology of NK cells.

2.4.3.1 Microcontact printing

The microcontact printing (μCP) technique involves transfer of molecules or proteins from an elastomeric PDMS stamp to a substrate surface and this technique has been used to pattern various proteins and cells on solid substrates (117). In paper IV, we used μCP to pattern ligands into arrays of AIS on glass surfaces. The μCP procedure is illustrated in figure 7. Microstructured stamps were made by casting and curing PDMS on a silicon master that held

the inverse pattern of the resulting stamps. Next, PDMS stamps were inked with a protein solution and dried. Finally the stamps were placed on the glass-bottom of a petri dish, with the structured surface facing the glass, and were left under weights for an hour. The stamp was then removed leaving a micro-sized pattern of protein on the glass.

2.4.3.2 Master fabrication

Silicon masters serving as a mold for the PDMS stamps were produced by photolithography and anisotropic etching. A photoresist was spin-coated onto a silicon wafer and then by exposing the photoresist to UV light through a photo mask followed by exposure to a solution of developer, the main structures of the master were formed. Further etching down into the silicon wafer was performed to make the structures higher. The photoresist was then removed by oxygen plasma cleaning and a C_4F_8 coating was made on the surface to facilitate release of the PDMS slab from to the master.

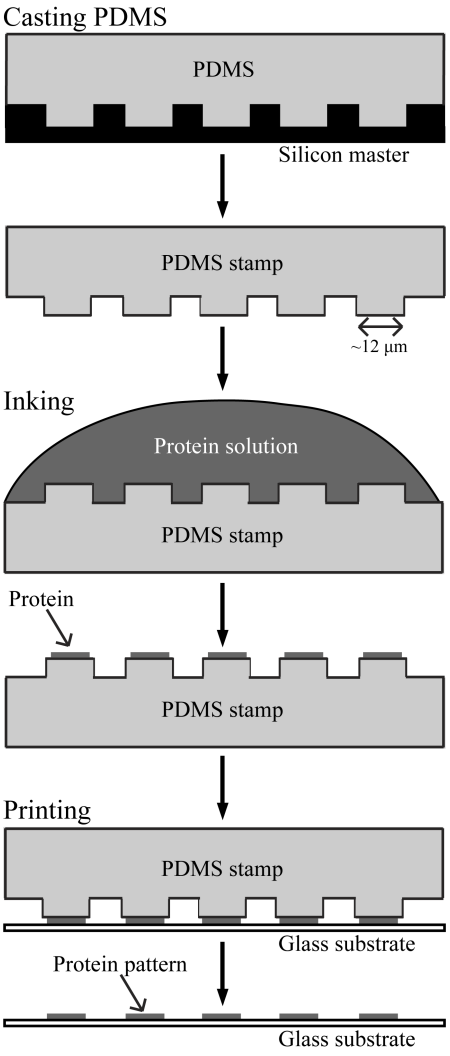

Figure 7. Microcontact printing of proteins was performed by casting PDMS on a microstructured silicon master, followed by inking with a protein solution and printing protein on a glass surface.

2.5 IMAGE ANALYSIS

2.5.1 Analysis of cell-cell interactions

In microwell assays, target cells and NK cells were co-incubated and at any given time point during the assay, individual NK cells can be either interacting with target cells or be in a state of free migration (Figure 8). Conjugates were identified as periods when a NK cell and target cell were in contact for at least 2 time points when the interface between the NK cell and target cell membranes appeared flat. The outcome of each NK-target cell interaction was scored as a kill or no kill, adding up to a total number of conjugates and kills for every NK cell. The end of the conjugate period and start of the attachment period was identified as a change in the morphology of the NK cell from a round to a elongated, migratory morphology, as illustrated in figure 8. In order to quantify the length of the conjugation and attachment periods, 3 time points were documented for each interaction: start of conjugation, start of attachment period, and detachment representing the end of the interaction (Figure 8).

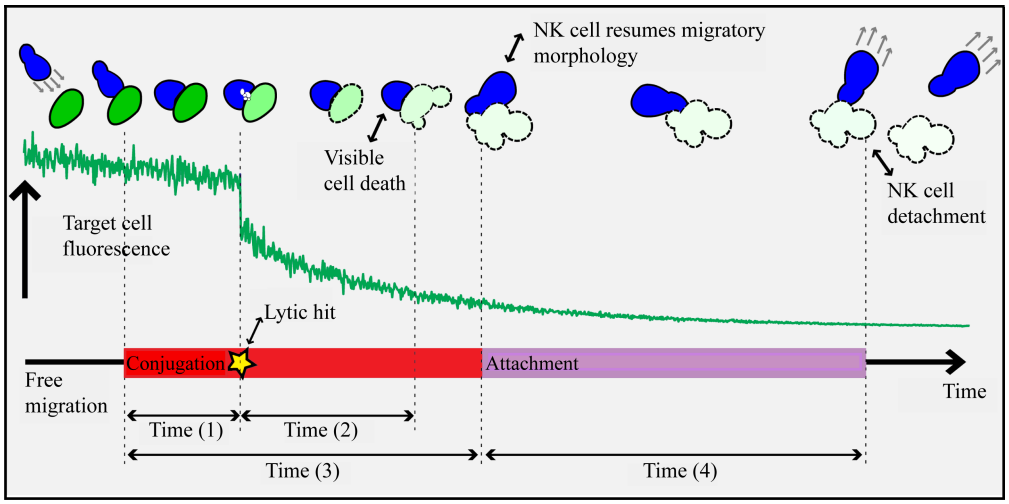

Figure 8. Identified events and associated time points during a NK-target cell interaction. The conjugate period starts as the migrating NK cell (blue) forms a tight conjugate with the target cell (green). Time (1) indicates duration until the lytic hit, which causes leakage of calcein and is consequently detected as a distinct drop in the calcein fluorescence intensity from the target cell. Time (2) indicates duration from the lytic hit until signs of death, typically membrane blebbing, can be seen. Time (3) indicates the duration of the conjugation period which ends as the NK cell resumes a migratory morphology. Time (4) indicates the duration of the attachment period, which is ended when the NK cell detaches from the target cell. Figure is adapted from paper I.

2.5.1.1 Estimating NK cell spreading at the immune synapse

When interacting with a target cell, formation of a lytic immune synapse is characterized by NK cell flattening and spreading, leading to an increased synapse diameter (48, 51). A similar NK cell spreading response has also been observed upon engagement of activating receptors presented on a substrate (116). In paper IV, this spreading response of NK cells in conjugates was measured by calculating the fraction of the NK cell membrane in contact with the target cell. Here the NK cell perimeter as well as the part of the membrane involved in the intercellular contact was outlined and the ratio interface/perimeter was calculated.

2.5.1.2 Detection and analysis of target cell death

As described in section 2.1, the intensity of the fluorescence emitted from cells stained with calcein dyes serves as an indication of viability. The intensity of calcein was here examined to identify killing events. Moreover, changes in morphology observed in the transmitted light channel, such as membrane blebbing or rupture, were also considered signs of target cell death. The fluorescence profile of calcein was analyzed in paper I in order to identify killing events as well as categorize target cell deaths as either fast or slow deaths. In papers II and III, target cell death was determined manually based on the fluorescence intensity of calcein as well as on visible signs of death.

2.5.1.3 Identifying lytic hits

In paper I, a lytic hit refers to the specific time point during a NK-target cell conjugation period when the content of the lytic granules has a detectable effect on the target cell

membrane that results in leakage of the calcein dye, as illustrated in figure 8. A lytic hit is thus identified in target cells as a drop in the total fluorescence intensity of the calcein dye.

2.5.2 Analysis of migration behavior

NK cells confined in microwells were tracked using a manual tracking tool in the image analysis software Volocity. For every time point in the collected time-lapse, the lateral (x,y) position for each NK cell was determined resulting in a trajectory for every tracked NK cell. Any NK cells that were dead, as evident by the lack of intracellular calcein staining, as well as clusters of NK cells, were excluded from the analysis. The trajectories were further analyzed by in-house developed tools using Matlab software. In this analysis the mean-square displacement (MSD) of the tracked NK cells was evaluated, which gives a description of the extent of the migration. Each trajectory was analyzed by a sliding window approach where parts of the trajectory, divided into sections of 25 time points, were assessed by sequentially moving the analysis-window one step forward per time point. The MSD was used to determine the diffusion coefficient, which is here used to analyze migration behavior and is therefore called the migration coefficient (M). The MSD can be written as:

$$MSD(t) = 4Mt^{\alpha}$$

Periods of low motility, called transient migration arrest periods (TMAP; (118)), were identified when the value of the migration coefficient was below a set threshold. Apart from TMAP, two other modes of migration was used to describe the migration behavior of NK cells, directed migration and random migration. In periods of directed migration, the NK cell typically migrates in a persistent direction whereas in random migration the behavior is similar to Brownian motion, *i.e.* corresponding to random oscillations with no preferred direction of a NK cell-sized particle. Furthermore, the value of α in the above equation was used to distinguish between directed and random migration behavior. The analysis resulted in that each trajectory from single NK cells was divided into periods of: 1) directed migration, 2) random migration, and 3) TMAP.

2.5.3 Evaluating cell morphology

Migrating NK cells often display a more elongated and assymmetric morphology than NK cells engaged in a conjugate which display a more round shape. In the present investigations the area and perimeter of NK cells were measured in ImageJ software and the roundness was calculated according to:

$$\text{Roundness} = \frac{4\pi \times \text{Area}}{\text{Perimeter}^2}$$

Hence the roundness of a perfectly round cell is 1 while a migrating, elongated cell will have a smaller roundness value.

2.5.4 Determining the lateral position of the MTOC in NK cells on AIS

The lateral distance from the MTOC to the center of patterned AIS was determined by first identifying NK cells that were in contact with AIS and which had spread out over the AIS, displaying a symmetric morphology and also had their MTOC polarized towards the glass surface. NK cells were thus chosen for analysis if the distance from the center of the cell to the center of the AIS was < 2 μm and if they had a roundness > 0.7. This selection was made in order to make sure that analyzed NK cells were definitely interacting with AIS. The lateral position of the MTOC was then identified as the largest cluster of α-tubulin.

3 AIMS

The overall aim of this thesis work was to study the functional heterogeneity within NK cell populations as well as to gain knowledge on the functional implications of NK cell education on a single cell level.

The specific aims were:

1. To characterize the heterogeneity within NK cell populations in terms of the cytotoxic response against tumor target cells.
2. To investigate what aspects of conjugate formation, killing and migration that are influenced by IL-2 activation of NK cells.
3. To reveal the functional implications of NK cell education in terms of conjugation and killing dynamics as well as migration behavior.
4. To study how the spatial distribution of activating ligands influence NK cell response.

4 RESULTS

The aims of this thesis were to perform single-cell analysis of the functional heterogeneity within NK cell populations and to contribute to further understanding of the functional implications of NK cell education. Moreover, we set out to study the responses of NK cells to spatially segregated activating ligands.

4.1 PAPER I: HETEROGENEITY IN THE CYTOTOXIC RESPONSE OF IL-2 ACTIVATED NK CELLS

In the experiments described in paper I, IL-2 activated NK cells confined in microwells with HEK293T target cells were followed by time-lapse imaging for 12 hours. The interactions of single NK cells with target cells were analyzed. Here the concepts of conjugation and attachment, which are also used in papers II and III, were defined as follows: 1) When the NK cell is in “conjugation” with a target cell, a tight contact is formed with a flat membrane-interface between the NK cell and target cell and 2) as the contact progresses and the target cell is either killed or spared, the NK cell attempts to migrate away while remaining in contact with the target cell, a state which is here called “attachment”. Conjugation and attachment phases are illustrated in figure 8, section 2.5.1.

4.1.1 Fast and slow target cell death

Duration of conjugation periods ranged from a few minutes to up to several hours. Lytic conjugation periods, which resulted in killing, were in general shorter than non-lytic conjugation periods. It was observed that in lytic conjugations, target cells could die either through a fast or slow process, as determined by the rate of decay of the calcein fluorescence intensity. Fast death appeared to be associated with rapid bursting of the plasma membrane while slow death was associated with membrane blebbing. It was hypothesized that slow death was caused by apoptosis-inducing granzymes whereas fast death was caused by high concentrations of perforin. To test this hypothesis, HEK293T target cells labeled with calcein were treated with melittin, a protein found in bee venom that is structurally similar to perforin and also forms pores in cell membranes (119-121). These experiments thus aimed to characterize the calcein intensity decay in target cells that died as a result of pore-formation in the plasma membrane. The effect of melittin on calcein leakage was found to be concentration-dependent. Moreover, melittin induced death caused a rate of decay in calcein intensity and signs of membrane bursting similar to that observed in NK cell-mediated fast death.

4.1.2 Delivery of lytic hits

Lytic hits were identified as a drop in the intensity of intracellular calcein. Most lytic hits were delivered within the first 20 minutes past onset of conjugation and 99% of target cells that had received a lytic hit later died. Although only one lytic hit was detected in most lytic interactions, NK cell could deliver multiple lytic hits during one conjugation period.

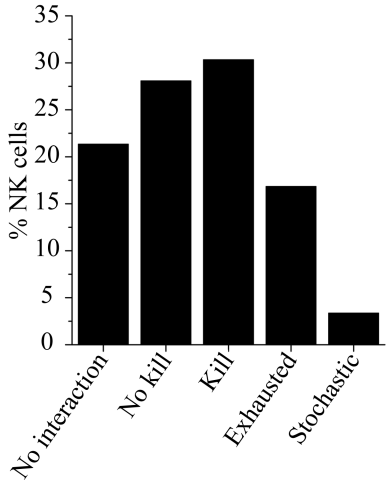

Figure 9. Classification of NK cells based on migration and cytotoxic response. The percentage of all NK cells in each group is shown. Figure is adapted from paper I.

4.1.3 Heterogeneity in the cytotoxic response

The number of contacts and killed target cells was quantified for all individual NK cells. The results revealed that 49% of all NK cells did not interact with target cells or kill any target cells (Figure 9). Instead a small group of NK cells was found to be responsible for most of the killing events.

4.1.3.1 Classification of NK cells based on their migration, contact and killing history

Based on the number of contacts and kills as well as the sequence of non-lytic and lytic interactions, NK cells could be divided into 5 different classes: 1) NK cells that never encountered target cells, 2) NK cells that never killed, 3) NK cells that always killed, 4) exhausted NK cells that first killed conjugated target cells but then failed to kill subsequently encountered target cells, and 5) NK cells killed or failed to kill conjugated cells in a stochastic manner. The percentage of NK cells in each class are shown in figure 9.

4.1.4 Features of serial killers

Approximately 20% of all NK cells killed ≥ 3 target cells and about 6% killed ≥ 5 target cells. Serial killers, defined in paper I as NK cells killing ≥ 5 target cells, could be distinguished from other killing NK cells in terms of conjugation and killing dynamics as they: 1) were more briefly conjugated to target cells 2) delivered their lytic hits faster, 3) more often caused fast target cell death, and 4) had a larger cell area.

4.2 PAPER II: COMPARISON OF MIGRATION AND KILLING DYNAMICS DISPLAYED BY RESTING AND IL-2 ACTIVATED NK CELLS

As a continuation of paper I, the work presented in paper II aimed to compare the conjugate formation, killing and migration behavior displayed by IL-2 activated NK cells to that of non-activated, resting NK cells. Experiments were carried out in a similar way as in paper I, resting NK cells or NK cells cultured in IL-2 for 7-16 days were confined in microwells with HEK293T target cells and imaged for 8 hours. The migration behavior of individual NK cells and their interactions with target cells were subsequently analyzed. In total, 221 IL-2 activated NK cells and 265 resting NK cells were analyzed.

4.2.1 IL-2 activated NK cells exhibit more dynamic migration behavior

The mean migration speed of single NK cells was found to be in the range of 1-3 μm/min for both NK cell populations although the mean speed of activated NK cells was slightly higher than the mean speed of resting NK cells (Table 2).

The obtained trajectories of single NK cells were divided into three modes of migration: 1) TMAP, 2) directed migration and 3) random migration (as described in section 2.5.2.) revealing differences in the migration behavior of resting and IL-2 activated NK cells. While resting NK cells typically spent almost the entire assay in TMAPs, activated NK cells spent less time in TMAPs and more time in directed and random migration modes. In addition, activated NK cells more often alternated between different modes of migration than resting NK cells (on average 2.8 vs. 1.1 alternations per NK cell; Table 2). Frequent alternations between different migration modes imply a dynamic stop-and-go migration behavior. Taken together, these results indicate that IL-2 treatment favors a migratory phenotype of NK cells.

4.2.2 Differences in cytotoxicity and conjugate formation

Only two killing events by resting NK cells were recorded suggesting that resting NK cells, in contrast to activated NK cells, do not readily kill HEK293T target cells. Comparing the frequency by which NK cells made contacts with target cells, it was found that activated NK cells formed more conjugates as compared to resting NK cells. Contacts made by activated NK cells also lasted longer than contacts made by resting NK cells, both in terms of conjugation and attachment periods.

Mean values	IL-2 activated NK cells	Resting NK cells
Migration speed	1.7 ± 0.7 μm/min	1.6 ± 0.6 μm/min
Number of alternations	2.8	1.1
Number of contacts	1.7	0.8

Table 2. Comparison of IL-2 activated and resting NK cells.

4.2.3 Differences in morphology

Outside of contacts with target cells, activated NK cells appeared more elongated and larger than resting NK cells and this observation was confirmed by evaluations of roundness and cell area. Moreover, whereas activated NK cells altered their morphology when engaging in contacts by assuming a more round shape and spreading across the target cell, resting NK cells did not display this behavior but instead maintained their already round shape in contacts.

4.3 PAPER III: FUNCTIONAL IMPLICATIONS OF NK CELL EDUCATION VIA NKG2A ON MIGRATION AND KILLING DYNAMICS

The results from the study in paper I revealed heterogeneity within NK cell populations in terms of contact and killing dynamics. It is known that the strength of the cytotoxic response is dependent on NK cell education (NK cell education is described in section 1.2.5) since NK cells which express cognate inhibitory receptors (IR) are more responsive than uneducated NK cells that lack expression of cognate inhibitory receptors (from here on called "IR^-"). Thus, the heterogeneity observed in paper I may be correlated to the variegated expression of KIR and NKG2A inhibitory receptors within NK cell populations. The work presented in paper III aimed to reveal what aspects of NK cell cytotoxicity that are influenced by the education status.

4.3.1 Effect of sorting and IL-2 activation on the cytotoxicity and phenotype of IR^- and $NKG2A^+$ NK cells

In order to study the functional implications of education on a single cell level, two distinct NK cell subsets at different levels of education were sorted: 1) $CD56^{dim}CD57^-KIR^-NKG2A^-$ (IR^-) and 2) $CD56^{dim}CD57^-KIR^-NKG2A^+$ ($NKG2A^+$) NK cells. $CD56^{bright}$ and $CD57^+$ NK cells were excluded in the sorting strategy in an attempt to sort subsets based on their education status and not their level of maturity. The sorted subsets were subsequently studied in microwell assays and flow cytometry-based assays.

4.3.1.1 Effect of FACS sorting on the cytotoxic response against K562 target cells

Assessing the percentage of cytotoxic NK cells within resting, non-sorted populations, the frequency of NK cells that killed K562 target cells in a 12 hour microwell assay was approximately 25%. These NK cells had been isolated with negative selection using magnetic beads. The IR^- and $NKG2A^+$ subsets were isolated by negative selection followed by sorting using FACS. The corresponding fractions of resting NK cells that killed K562 target cells in

sorted populations were 1% in both the IR^- and the $NKG2A^+$ subset. Hence the sorting process resulted in a smaller fraction of NK cells that killed K562 target cells in microwells. Therefore both subsets were maintained in IL-2 for two days prior to microwell experiments in order to restore the weakened cytotoxic response imposed by the sorting process.

4.3.1.2 Effect of IL-2 activation on the cytotoxicity and degranulation response to K562 target cells

After 2 days culture in IL-2, the frequency of killers was higher in the $NKG2A^+$ subset as compared to the IR^- subset (Figure 10A). However, after 7 days culture in IL-2, the frequency of killers was similar in both subsets. Moreover, the frequency of degranulating $NKG2A^+$ and IR^- NK cells was measured by flow cytometry by assessing the expression of $CD107a^+$ on NK cells subsequent of co-incubation with target cells. In consistency with the observed IL-2 induced increase in the frequency of cytotoxic IR^- NK cells (Figure 10A), the frequency of degranulating IR^- NK cells also increased with 2 days IL-2 activation (Figure 10B). However, the increase in degranulating IR^- NK cells was more dramatic than the corresponding increase in cytotoxic IR^- NK cells. Moreover, while the frequency of cytotoxic $NKG2A^+$ NK cells increased considerably with 2 days IL-2 activation (Figure 10A), there was no clear corresponding effect on the frequency of degranulating $NKG2A^+$ NK cells (Figure 10B). In addition, while only 1% of the resting IR^- or $NKG2A^+$ NK cells killed K562 target cells in microwell experiments (Figure 10A), the frequencies of degranulating resting NK cells were on average 4% of IR^- NK cells and 40% of $NKG2A^+$ NK cells (Figure 10B). Thus the indicated frequencies of cytotoxic resting IR^- and $NKG2A^+$ NK cells from degranulation assays did not correlate well with the corresponding results from microwell assays.

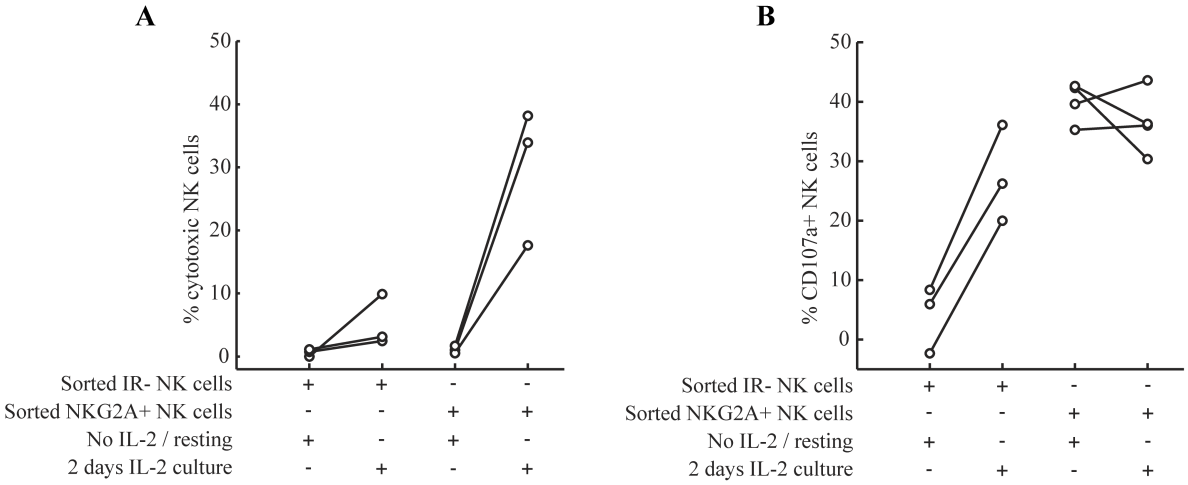

Figure 10. Killing of K562 in microwells and degranulation response to K562 measured by flow cytometry. (**A**) Frequencies of IR^- and $NKG2A^+$ NK cells, resting or cultured for 2 days in IL-2, that killed K562 target cells in microwells. (**B**) Frequencies of IR^- and $NKG2A^+$ NK cells, resting or cultured for 2 days in IL-2, that degranulated ($CD107a^+$) in response to K562 target cells. Shown values in B have been corrected by withdrawing frequencies from unstimulated cultures. Total number of analyzed resting NK cells in microwell assays were 335 resting and 441 IL-2 activated IR^- NK cells and 408 resting and 751 IL-2 activated $NKG2A^+$ NK cells. Figure is adapted from paper III.

4.3.1.3 Effects of IL-2 activation on phenotype and function of IR$^-$ and NKG2A$^+$ NK cells

The expression levels of NKG2A, KIR and CD57 were evaluated by flow cytometry before and after IL-2 culture in order to estimate phenotypic changes induced by IL-2. The results showed that NKG2A expression was induced in approximately 8% of the IR$^-$ NK cells. Moreover, a few percent of both IR$^-$ and NKG2A$^+$ NK cells acquired KIR and CD57 expression. As acquisition of NKG2A and KIR expression could be associated with an increase in the cytotoxic response, degranulation assays were performed in order to determine the contribution of IR$^-$ NK cells that had acquired NKG2A or KIR and that of NKG2A$^+$ NK cells that had acquired KIR expression. The results indicated that the majority of the IR$^-$ NK cells that had degranulated in response to both K562 or HEK293T cells, had not acquired NKG2A or KIR. Similarly, a majority of the CD107a$^+$ NKG2A$^+$ NK cells had not acquired KIR expression. Thus, it appeared that IL-2 induced expression of NKG2A and KIR did not contribute significantly to the degranulation response in either subset. However, in the IR$^-$ subset, the frequency of CD107a$^+$ NK cells was higher among those that had acquired expression of NKG2A, suggesting that these cells could be more likely to kill target cells.

4.3.2 Migration behavior, contact and killing dynamics displayed by IR$^-$ and NKG2A$^+$ NK cells

Sorted IR$^-$ and NKG2A$^+$ NK cells were incubated separately with HEK293T target cells in 450×450×300 μm^3 microwells or with K562 target cells in 50×50×300 μm^3 (Image of 81 microwells is shown in figure 11A), and were followed with time-lapse imaging for 12 hours. The migration behavior of 102 IR$^-$ NK cells and 101 NKG2A$^+$ NK cells as well as their interactions with HEK293T target cells were subsequently analyzed. Conjugates were scored as lytic or non-lytic depending on outcome, an image-sequence of a NK cell killing a K562 target cell in a microwell is shown in figure 11B.

4.3.2.1 NKG2A$^+$ NK cells displayed more dynamic migration behavior

There was no significant difference in terms of average migration speed between IR$^-$ (median 1.4 μm/min) and NKG2A$^+$ (median 1.6 μm/min) NK cells. An analysis of the migration behavior revealed that NKG2A$^+$ NK cells spent more time in random and directed migration and less time in TMAPs as compared to IR$^-$ NK cells. Furthermore, NKG2A$^+$ NK cells also more frequently altered between different modes of migration compared to IR$^-$ NK cells (on average 3.3 vs. 2.4 alterations per NK cell) and thus the results indicate that NKG2A$^+$ NK cells display a more dynamic migration behavior than IR$^-$ NK cells.

4.3.2.2 NKG2A$^+$ NK cells formed more conjugates and spent more time interacting with target cells

The subsequent analysis of NK cells interacting with HEK293T target cells revealed that NKG2A$^+$ NK cells more frequently formed conjugations with target cells than IR$^-$ NK cells. On average, NKG2A$^+$ NK cells made 2.6 contacts per NK cell while the corresponding number was 1.5 contacts per IR$^-$ NK cell. Moreover, in experiments with HEK293T target cells, IR$^-$ NK cells spent less time interacting with target cells compared to NKG2A$^+$ NK cells (on average 24% vs. 52%). There was also a higher fraction of killers and serial killers

(here defined as killing ≥ 3 target cells) among NKG2A$^+$ NK cells, as shown for HEK293T target cells in figure 11C.

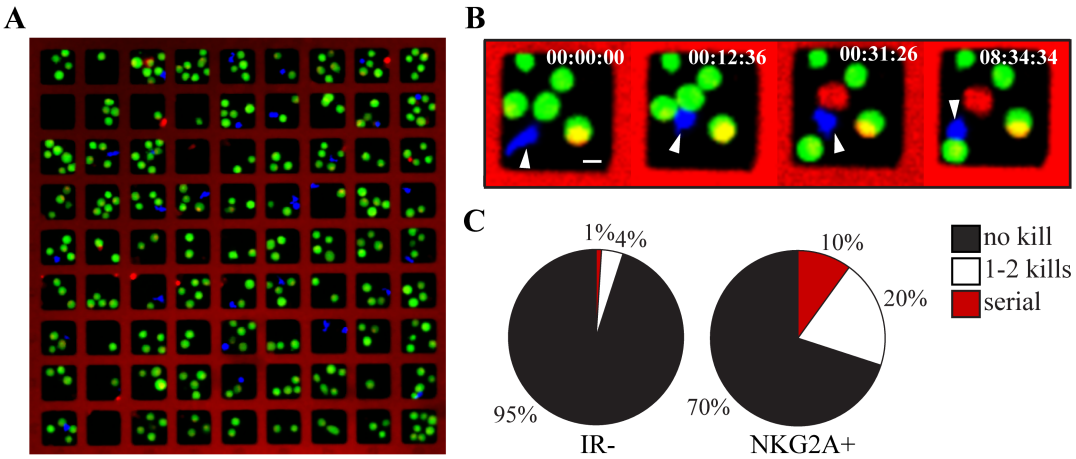

Figure 11. Killing of K562 target cells. (**A**) Image of 81 microwells with living (green) and dead (red) K562 target cells incubated with NK cells (blue) (**B**) Image-sequence of a microwell containing a NK cell that kills a K562 target cell (out of 5), target cell death is identified as loss of calcein (green) intensity while the intensity of DDAO (red) is sustained. (**C**) Pie charts showing frequencies of non-killers (black) NK cells that killed 1-2 target cells (white), and serial killers (red) that killed ≥ 3 target cells in IR$^-$ and NKG2A$^+$ NK cell populations. Total number of analyzed NK cells were N_{IR-}=382 and N_{NKG2A+}=575. Figure is adapted from paper III.

4.3.2.3 NKG2A$^+$ NK cells are more likely to kill conjugated target cells

A comparison of the fraction of killing conjugates showed that conjugates formed by NKG2A$^+$ NK cells were more likely to result in target cell death compared to conjugates involving IR$^-$ NK cells (Figure 12). This suggests that NKG2A$^+$ NK cells are more efficient killers when encountering target cells than IR$^-$ NK cells. Furthermore, the superior killing efficiency of NKG2A$^+$ NK cells was more pronounced in conjugates with K562 (on average 12% vs. 60% lytic conjugates respectively) compared to HEK293T (on average 23% vs. 50% lytic conjugates respectively) target cells (Figure 12).

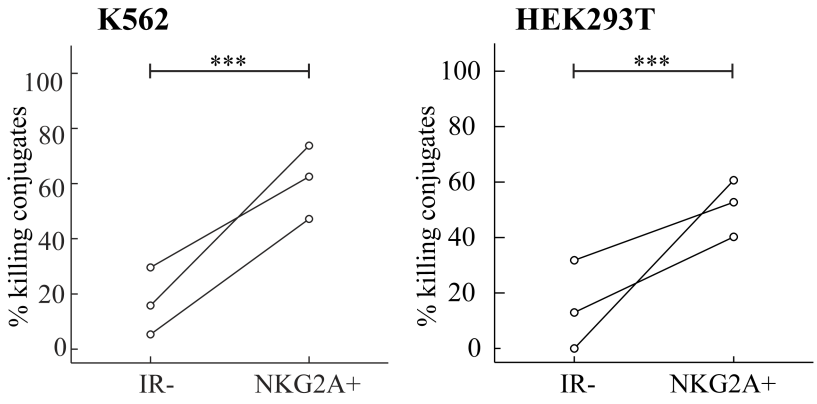

Figure 12. Fraction of conjugates leading to K562 and HEK293T target cell death, by IR$^-$ and NKG2A$^+$ NK cells. *** indicates statistical significance level $p < 0.001$. Figure is adapted from paper III.

4.3.2.4 NKG2A$^+$ and IR$^-$ NK cells exhibit similar killing dynamics

An evaluation of the duration of individual conjugation periods, divided into lytic and non-lytic interactions with HEK293T target cells, revealed that in non-lytic interactions, NKG2A$^+$ NK cells remained conjugated to target cells for longer periods than IR$^-$ NK cells. In contrast, duration of lytic conjugation periods did not differ between the two subsets. Still, the majority of the contacts made by IR$^-$ NK cells did not result in killing, suggesting that IR$^-$ NK cells were not sufficiently activated for killing to occur. In order to assess the level of activation we evaluated NK cell spreading in contacts and the results showed no difference between the two subsets in lytic conjugates but NKG2A$^+$ NK cells exhibited an increased spreading response in non-lytic conjugates. Taken together, these findings suggest that the small fraction of IR$^-$ NK cells that did kill target cells, did so in a similar manner as NKG2A$^+$ NK cells.

4.4 PAPER IV: ASSESSING NK CELL RESPONSES TO PATTERNED ARTIFICIAL IMMUNE SYNAPSES

NK cell cytotoxicity is a highly regulated process where the lytic immune synapse is formed in a stepwise manner (lytic synapse formation is described in section 1.2.2.2). Although the different steps leading to immune synapse formation have been at least partially revealed, it is still not clear if the spatial distribution of ligands influences the functional response of NK cells. In paper IV we aimed to assess NK cell responses to different activating ligands and determine the potential influence of their spatial distribution on a surface.

Antibodies or ligands targeting the LFA-1 adhesion receptor or the CD16 FcR alone as well as LFA-1 and CD16 mixed together, were patterned into distinct AIS on glass surfaces and the response of NK cells interacting with AIS were monitored and analyzed. Other studies have shown that ligation of LFA-1 initiates immune synapse formation and that clustering of LFA-1 and actin polymerization facilitate firm adhesion of the NK cell to the target cell (48, 53). Moreover, LFA-1 engagement has also been shown to stimulate NK cells to migrate and assume an elongated morphology (116).

Ligation of LFA-1 alone has been shown to induce polarization of lytic granules but not degranulation whereas ligation of CD16 was observed to stimulate degranulation but not polarization of lytic granules (52). The same study also showed that when both LFA-1 and CD16 were engaged, polarization and directed degranulation of lytic granules occurred (52). Furthermore, although the functional outcomes differ, the intracellular signals prompted by LFA-1 and CD16 ligation are similar (122). Still, whereas intracellular signaling triggered by CD16 results in Ca^{2+} mobilization, LFA-1 induced signals do not (122).

4.4.1 LFA-1 and CD16 induce different morphologies and migration responses

The responses of live NK cells interacting with AIS composed of antibodies for LFA-1 and CD16 were followed with time-lapse imaging. NK cells were observed to migrate on anti-LFA-1 AIS while contacts with anti-CD16 AIS instead made NK cells stop and spread over

the AIS. These observations were confirmed by tracking the movement of individual NK cells subsequent of their initial contact with AIS (Figure 13A). Moreover, the roundness of NK cells in contact with torus-shaped AIS was evaluated at a set time point. The results confirmed that anti-CD16 AIS stimulated a symmetric morphology in contrast to anti-LFA-1 AIS on which most NK cells most displayed an elongated morphology (Figure 13B-C). When both LFA-1 and CD16 were ligated, the observed morphology was similar to that observed when CD16 alone was ligated (Figure 13C). Furthermore, contacts with anti-LFA-1 AIS were often linked to the formation of nanotubes (123), *i.e.* membrane tethers connecting the NK cell to one or several AIS.

4.4.2 Spatial distribution of patterned ligands impact the stability of contacts

A quantification of the time duration of contacts with torus-shaped and dot-shaped anti-CD16 AIS revealed that the contact time seemed to be dependent on if the NK cell made a complete or partial contact with the AIS. Partial contacts were generally shorter and more common on torus-shaped patterns. Similar observations have been made in a previous study where T cells were imaged while interacting with micropatterned antibodies against the TCR (114). However, NK cells that spread out over the torus and thus made a complete contact appeared to provide a more stable adhesion that lead to a longer interaction. Moreover, NK cells loaded with the calcium indicator dye Fluo-4 that made stable contacts often displayed continuous intracellular calcium signaling for the entire six-hour experiment.

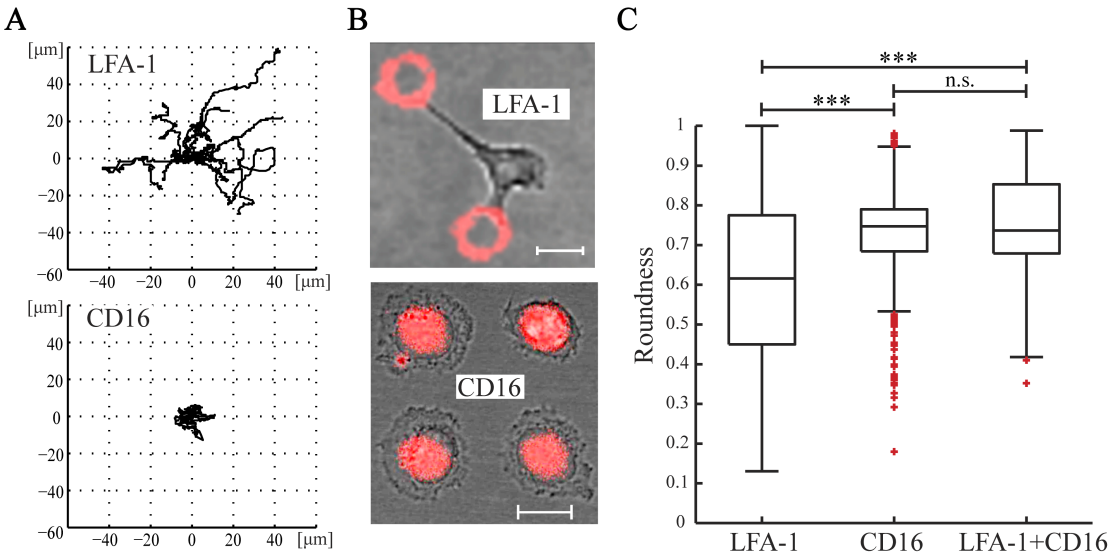

Figure 13. Contacts with anti-LFA-1 and anti-CD16 AIS stimulate different migratory and morphological responses. (**A**) Trajectories of NK cells that were tracked for 30 minutes from the initial contact with torus-shaped AIS of anti-LFA-1 and anti-CD16. N=15 cells per condition were tracked. (**B**) Representative images of NK cells in contact with torus-shaped anti-LFA-1 AIS and dot-shaped anti-CD16 AIS, (AIS are shown in red). Scale bars indicate 10 µm. (**C**) Box plot of roundness values of NK cells in contact with torus-shaped AIS of anti-LFA-1, anti-CD16, or both, where a perfectly round shape has roundness value 1. N_{LFA-1}= 214, N_{CD16}= 466 and $N_{CD16+LFA-1}$=370 NK cells. *** indicates statistical significance level $p < 0.001$. Figure is adapted from paper IV.

4.4.3 Lateral position of MTOC in NK cells on AIS

To assess if the spatial distribution of ligands impacts the lateral position of the MTOC, NK cells were incubated on glass substrates patterned with AIS composed of antibodies against both CD16 and LFA-1. The NK cells were subsequently fixed and stained for tubulin and imaged with confocal microscopy. The results indicated that the distance from the MTOC to the center of the AIS was similar in NK cells interacting with torus-shaped and dot-shaped AIS. The distance was approximately 2 μm on both types of AIS.

5 DISCUSSION AND FUTURE PERSPECTIVES

5.1 HETEROGENEITY IN THE CYTOTOXIC RESPONSE OF IL-2 ACTIVATED NK CELLS

The work described in paper I involved classification of IL-2 activated polyclonal NK cells according to their cytotoxic response. NK cells classified as stochastic killers made both lytic and non-lytic interactions in a seemingly random fashion. This group of stochastic killers was notably smaller than the groups of NK cells that consequently killed or failed to kill conjugated target cells, suggesting that NK cells generally exhibit binary commitment to either killer or non-killer groups. It can be speculated that non-killers perhaps function mainly through cytokine secretion. However, it has previously been shown that there is no difference in the levels of cytokine secretion of non-cytotoxic compared to cytotoxic NK cells (111).

The relatively long culture in IL-2 (9-16 days) before the experiments could possibly induce proliferation in some NK cell subsets while other subsets of NK cells may die during this time period. These processes could interfere with the cytotoxic function of NK cells and thus it would be of interest to investigate the occurrence of proliferation and cell death in relation to the cytotoxicity of individual NK cells.

5.1.1 Fast and slow death

NK cells could induce target cell death via a fast or slow process where a fast death process was accompanied by membrane bursting whereas target cells that died through slow processes displayed apoptotic membrane blebbing. Bursting of the cell membrane cause intracellular components to be released into the extracellular environment. These can promote inflammation that could results in damage to nearby cells and tissues, *e.g.* release of the high-mobility group protein box 1 protein which activates the innate immune system through binding of TLRs (124). In contrast, apoptosis is a more controlled death process in which the intracellular content is retained and cells that have died from apoptosis are normally phagocytosed. Thus, these two types of death processes can cause different consequences to cells in their surrounding environment. It would be interesting to investigate if the fast death process is only caused by cytokine activated NK cells or if cytotoxic resting NK cells also induce both types of death processes.

5.2 IDENTIFICATION OF SERIAL KILLERS

NK cells capable of killing several target cells successively, so called serial killers, have also been described previously (125). In paper I we defined serial killers as NK cells that killed ≥ 5 target cells and in paper III we had adjusted this definition to NK cells that killed ≥ 3 target cells in consensus with a previous study from our group (126). In a recent investigation assessing the ability of individual NK cells' to kill antibody-coated tumor cells via ADCC, serial killers were defined as NK cells killing ≥ 2 target cells (127). Because of their efficient killing of tumor cells, serial killers represent a potential candidate for cancer therapies that may involve clonal expansion and subsequent adoptive transfer of these NK cells. For such aims, serial killers need to be isolated from NK cell populations. These NK cells could then be further analyzed to determine not only their surface expression profiles but also functional features like their proliferation potential or cytokine secretion. Using the microwell assays presented in this thesis, isolation of NK cells based on their level of cytotoxic response is a long-term goal. There is an ongoing project in our group that aims to develop a mechanism for retrieving NK cells from the microwells subsequent of experiments. Furthermore, by measuring the cell area of single NK cells, we noticed that serial killers have a larger cell area compared to the other NK cells. Thus an alternative approach to isolating serial killers could be to sort out the largest NK cells from IL-2 activated populations.

5.3 EFFECT OF IL-2 ACTIVATION ON MIGRATION, CONJUGATE FORMATION AND CYTOTOXICITY

The findings in paper II showed that IL-2 had an effect on the migration behavior as IL-2 activated NK cells displayed a more migratory morphology, spent more time migrating and also more frequently alternated between different modes of migration compared to resting NK cells. The majority of resting NK cells did not make any contacts with target cells and only two HEK293T target cells were killed by resting NK cells. The reduced frequency of resting NK cells that made contacts with target cells is likely coupled to the impaired migration behavior displayed by these cells since NK cells usually need to migrate in order to find target cells in the microwells. Also, conjugates made by IL-2 activated NK cells lasted for longer time periods than conjugates involving resting NK cells, suggesting that the ability to form stable conjugates is diminished in resting NK cells.

Most IL-2 activated NK cells described in paper I and II killed HEK293T target cells. Also the results in paper III showed that most IL-2 activated NKG2A$^+$ NK cells killed HEK293T target cells. Resting NK cells did not however kill HEK293T target cells. This could be due to inhibitory input received via receptors interacting with HLA present on the surfaces of HEK293T target cells. In contrast, killing of HLA negative K562 target cells by resting NK cells was observed in experiments presented in paper III where it was found that 25% of non-sorted resting NK cells killed K562 target cells in microwell experiments. Thus it appears that the cytotoxic response of resting NK cells is more target cell specific than that of IL-2 activated NK cells. A more comprehensive study of resting NK cells assessing conjugate formation and killing of K562 target cells could provide some additional insights to the

distribution of cytotoxic potential in resting NK cell populations. Also, since it has been shown that educated NK cells display better survival compared to non-educated NK cells (75), an investigation of the relative proliferation and survival of sorted IR^- and $NKG2A^+$ NK cells in response to IL-2 or IL-15 could help us understand how these subsets respond to cytokine treatment.

5.4 IMPLICATIONS OF EDUCATION ON ASPECTS OF NK CELL CYTOTOXICITY

The concept of NK cell education or licensing refers to a process that regulates the responsiveness of NK cells, that is the ability of NK cells to respond to stimuli by performing cytokine secretion or cytotoxicity. Moreover, the level of education correlates with the expression of cognate inhibitory receptors and thus the functional responsiveness can be predicted to some extent by the expression of inhibitory receptors, assuming that the host expression of inhibitory ligands is known.

5.4.1 Heterogeneity in the cytotoxic response within IR^- and $NKG2A^+$ NK cell populations

In paper I we observed a significant heterogeneity in the cytotoxic response within polyclonal NK cells populations. This heterogeneity could at least partially be explained by that individual NK cells within these populations were at different levels of education. In the experiments presented in paper III, we sorted non-educated and educated NK cell subsets. Here mature $CD56^{dim}CD57^-KIR^-$ NK cells were sorted based on their expression of the inhibitory receptor NKG2A. The cytotoxic responses of sorted IR^- and $NKG2A^+$ NK cells were characterized and compared. In consistency with previous studies (88, 89), the frequency of cytotoxic NK cells was higher in $NKG2A^+$ populations compared to IR^- populations. Still, we observed a heterogeneity in the cytotoxic response among both IR^- and $NKG2A^+$ NK cells. A small fraction of IR^- NK cells killed target cells and thus not all IR^- NK cells were hyporesponsive. It could be argued that the IL-2 treatment, which apparently induced expression of NKG2A or KIR in some cells, also made these cells cytotoxic. Still, a small fraction of resting IR^- NK cells degranulated in response to target cells indicating that these cells may be able to exert cytotoxicity also in the absence of cytokine activation. Conversely, large fractions of $NKG2A^+$ NK cells did not upregulate CD107a in degranulation assays or kill target cells in microwell assays. Unfortunately, we were not able to assess the expression profile of sorted NK cells subsequent of the microwell assays. Such analysis could have revealed if the IR^- NK cells that killed target cells had acquired expression of NKG2A or KIR.

5.4.1.1 Inhibition and activation of IR^- and $NKG2A^+$ NK cells in conjugates with K562 and HEK293T target cells

The outcome of each NK-target cell conjugation is directed by a balance of activating and inhibitory signals received by the NK cell. The observed difference in killing between the IR^- and $NKG2A^+$ subsets was more pronounced in the experiments with K562 compared to

HEK293T target cells. A possible explanation to this is that while HEK293T cells express low levels of HLA, K562 target cells are HLA negative. The $NKG2A^+$ but not the IR^- NK cells should thus receive some inhibitory signals via NKG2A-HLA-E interactions in conjugates with HEK293T cells.

The finding that $NKG2A^+$ NK cells were more likely to kill conjugated target cells raises the question of which receptor-ligand interactions that direct killing in these NK-target cell conjugates. The more efficient killing by $NKG2A^+$ NK cells as compared to IR^- NK cells could be due to higher expression levels of activating receptors on $NKG2A^+$ NK cells. Preferably we would like to map the expression of various activating receptors on sorted IR^- and $NKG2A^+$ NK cells before and after IL-2 treatment. However, because of the small populations of cells obtained from FACS sorting, we did not have enough cells to completely determine the expression of activating receptors on the subsets. Still, we included analysis of DNAM-1 and NKG2D expression since ligands for DNAM-1 are expressed on K562 cells and HEK293T express ligands for DNAM-1 and NKG2D (data not shown). We found no difference in the level of NKG2D expression but the expression level of DNAM-1 was higher on $NKG2A^+$ NK cells compared to IR^- NK cells, both in a resting state and after 2 days IL-2 activation (data not shown).

A higher expression of DNAM-1 on educated NK cells has been observed previously (78) and we have also recently shown that DNAM-1 expression correlates with the level of education (128). Thus it is likely that recognition via DNAM-1 has a central role in conjugate formation and killing of K562 and HEK293T target cells. A suitable strategy aiming to reveal which particular receptor-ligand interactions that direct killing of HEK293T and K562 by IR^- and $NKG2A^+$ NK cells would be to investigate the effect of antibody-mediated blocking of activating receptors in degranulation and microwell killing assays.

5.4.2 Correlation between frequencies of CD107a+ NK cells and cytotoxic NK cells

In paper III the observed frequencies of resting IR^- and $NKG2A^+$ NK cells that killed K562 target cells in microwell assays did not correlate well with the corresponding frequencies of degranulating NK cells as determined by CD107a assays (Figure 10). The frequencies of degranulating resting NK cells was considerably higher than the observed frequencies of resting NK cells that killed K562 target cells. The dissimilarities in the obtained results from these two different assays could be partially explained by discrepancies in the experimental setup. In microwell assays, NK cells most often need to migrate in order to contact target cells while in degranulation assays all NK cells are presumably in contact with target cells. This could influence the results since resting NK cells exhibit restricted migration. Thus it could be that a lesser fraction of NK cells made contacts with target cells in the microwell assay as compared to the degranulation assay. This is also reasonable when considering that the frequencies of degranulating and cytotoxic NK cells in response to K562 were more comparable after 2 days of IL-2 activation (40% degranulating vs. 30% cytotoxic NK cells), as the IL-2 activation presumably also has made the NK cells more motile.

However, although expression of CD107a correlates with cytotoxicity, it also correlates with cytokine secretion (106). Whereas the microwell assays presented here provide an experimental approach to directly measure the cytotoxicity of single NK cells, measurements of CD107a expression gives a less direct measurement that estimates the activity of NK cells. Moreover, considering that the toxic effect of the pore-forming protein melittin was concentration-dependent, it is reasonable to assume that the quantity of perforin delivered into the immune synapse through degranulation influences the resulting toxic effect on the target cell. Accordingly, NK cells that degranulate do not necessarily cause target cell death. Hence, although CD107a expression can be used to estimate the frequency of responsive NK cells within a population, it should not be assumed to be a direct measurement of the frequency of cytotoxic NK cells.

5.5 NK CELL RESPONSES ON ARTIFICIAL IMMUNE SYNAPSES

The results described in paper IV were obtained from experiments where NK cells were imaged while interacting with ligands patterned into spatially separated AIS. The patterned AIS were composed of antibodies or ligands that engaged LFA-1 or CD16 on NK cells. The results showed that ligation of LFA-1 induced a migratory response while NK cells interacting with anti-CD16 AIS, stopped and spread out over the AIS, assuming a symmetric morphology. Furthermore, NK cells more often made a complete contact, where the entire AIS was covered by the NK cell, on dot-shaped AIS as compared to torus-shaped AIS. Hence it appears that formation of complete, stable contacts were more difficult to achieve on torus-shaped AIS. Spreading over anti-CD16 AIS was linked to a continuous intracellular Ca^{2+} flux and this sustained activation of the NK cell could possibly assist in maintaining the symmetric shape of the NK cell and help stabilize the contact. Accordingly, the spatial distribution of anti-CD16 has an effect on the spreading of NK cells contacting the AIS. Thus, it is possible that the spatial distribution of ligands presented on target cell surfaces can influence NK cell spreading and the stability of the formed conjugate.

The position of the MTOC relative to the center of the AIS was determined in NK cells interacting with dot-shaped or torus-shaped AIS composed of antibodies against both CD16 and LFA-1. The results did not indicate any apparent differences in the positioning of the MTOC on the different types of AIS. The MTOC was on both types of AIS positioned slightly off-center, suggesting that the MTOC can be directed to an area that lacks local stimuli. However, these results are based on few experiments and further studies are needed in order to reveal to what extent the positioning of the MTOC and lytic granules is directed by the spatial distribution of ligands.

6 CONCLUDING REMARKS

We have shown that NK cells are heterogenous in terms of the frequency by which they contact and kill tumor cells. Most NK cells that were assessed here displayed a binary commitment as they were dedicated to a 'kill' or 'no kill' type of behavior.

It was found that NK cells could kill target cells via a fast or slow process. Fast death and accompanying membrane bursting, which is likely triggered by a high concentrations of perforin, could result in damage to surrounding cells and tissues. It would be of interest to determine under which conditions this type of killing occurs, such as if cytokine activation of NK cells is required.

A central aim in the further development of the microwell assays presented in this thesis is to enable isolation of NK cells based on their level of cytotoxic response, in particular serial killers that could then be characterized in terms of surface-expression or expanded for subsequent use in cancer therapies. Working towards this aim, an ongoing project aims to develop a technical solution that would enable recovery of cells from the microwells and thus facilitate isolation of serial killer populations.

Resting NK cells displayed a reduced ability to migrate and form conjugates compared to IL-2 activated NK cells. Moreover, although most IL-2 activated NK cells killed HEK293T target cells, resting NK cells did not. Further microwell studies of resting NK cells incubated with K562 target cells or other types of target cells could provide additional insights to the distribution and regulation of cytotoxicity in resting NK cell populations.

Heterogeneity in the migration behavior and cytotoxic response was observed within defined subsets of uneducated IR^- and educated $NKG2A^+$ NK cells. Intriguingly, serial killers were observed within the IR^- subset. Still, the frequencies of NK cells that formed conjugates and killed target cells were significantly higher among $NKG2A^+$ compared to IR^- NK cells. Hence, the overall relative strength of the cytotoxic response of these two subsets could be predicted by their respective levels of education.

It is possible that recognition via DNAM-1 on NK cells has a central role in conjugate formation and killing of K562 and HEK293T target cells. The higher expression level of DNAM-1 on $NKG2A^+$ NK cells compared to IR^- NK cells could thus contribute to the superior ability of $NKG2A^+$ NK cells to form conjugates and kill these target cells. Future work should therefore aim to resolve to what extent specific activating receptor-ligand interactions direct killing of these target cell lines in microwell killing assays.

We have found that that ligation of LFA-1 and CD16 through interactions with AIS induced different morphologies and migration responses in NK cells. In addition, the spatial distribution of ligands influenced the spreading of NK cells over anti-CD16 AIS, indicating that the spatial distribution of ligands on target cells can influence the stability of formed conjugates. Further studies should focus on revealing to what extent the positioning of the MTOC and lytic granules is directed by the spatial distribution of ligands.

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